# The Journal of Comparative Pathology and Therapeutics

Vol. 69. No. 2 Price Seventeen Shillings and Sixpence

CONTENTS

PAGE

Studies on the Antigenic Structure of *Vibrio fetus*. By W. J. Brinley Morgan .. .. .. .. .. .. 125

Goat Pox in Iran. Serial Passage in Goats and the Developing Egg, and Relationship with Sheep Pox. By A. Rafyi and H. Ramyar 141

Mortality of Rabbits Inoculated with Lapinised Rinderpest Virus. By G. R. Scott .. .. .. .. .. .. .. 148

Studies with Rinderpest Virus in Tissue Culture. I. Growth and Cytopathogenicity. By W. Plowright and R. D. Ferris .. 152

Studies with Rinderpest Virus in Tissue Culture. II. Pathogenicity for Cattle of Culture-Passaged Virus. By W. Plowright and R. D. Ferris .. .. .. .. .. .. .. 173

Some Observations on the Histopathology of Aldrin Poisoning in Lambs. By J. T. Baxter .. .. .. .. .. 185

The Effects of Different Anticoccidial Agents on the Intestinal Coccidioses of the Fowl. By C. Horton-Smith and P. L. Long 192

Staphylococcal Antitoxin in the Blood and Milk of Cattle. By S. J. Edwards and G. S. Smith .. .. .. .. 208

Observations on the Effects of Gonadectomy and Subsequent Injection of Testosterone on the Adrenals of Male Sheep. By W. M. Stokoe .. .. .. .. .. .. 216

Familial Cerebellar Degeneration and Atrophy—a Sex-Linked Disease Affecting Light Sussex Pullets. By L. M. Markson, R. B. A. Carnaghan and G. B. Young .. .. .. 223

Studies on *Pasteurella septica*. III. Strains from Human Beings. By J. E. Smith .. .. .. .. .. .. .. 231

# STUDIES ON THE ANTIGENIC STRUCTURE OF *VIBRIO FETUS**

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## INTRODUCTION

An impetus to studies on the antigenic structure of *Vibrio fetus* has recently been given in order to obtain satisfactory agglutinating antigens for sero-diagnostic work. Both in sheep and in cattle, it has repeatedly been found, that, depending on the antigen used, the titres in the serum agglutination test varied considerably. This observation has suggested the existence of serological groups within the species.

In the present work, a large number of ovine and bovine strains of *V. fetus* from various countries was collected. They were accepted as *V. fetus* if they satisfied the following conditions:—(a) they required added $CO_2$ for growth on solid media; (b) they were catalase positive, $H_2S$ negative and reduced nitrates to nitrites. The purpose of the investigations was to determine whether members of such a collection formed a serologically homogeneous group, or whether distinct serological groups occurred, and further to determine which antigens were group specific and which were type specific.

## METHODS

*Strains.* The strains used in this study were isolated during the diagnostic work already reported (Morgan 1956; Morgan, Melrose and Stewart, 1958), together with ovine and bovine strains received from workers in this country, the U.S.A. and Denmark. All the strains were routinely cultured in Brewer's medium and subcultured approximately every four weeks. Later, they were freeze-dried in "Mist desiccans" containing serum, glucose and Hartley broth (Fry and Greaves, 1951). They required added $CO_2$ for growth on solid media, were catalase positive and reduced nitrates to nitrites; the majority did not produce $H_2S$ although some caused a blackening of the tip of the lead acetate paper. Practically all such strains were old laboratory cultures.

*Preparation of antigens.* Both agglutinating and immunizing antigens were prepared by seeding cultures from Brewer's medium on to Thio agar in Roux flasks. These were then incubated in 10 per cent $CO_2$ for four days after which each flask was examined for purity and for motility.

For OH antigens, the growth from the Roux flasks was suspended in 0·3 per cent formol saline with the aid of sterile glass beads. The

*A condensation of part of a thesis submitted to the University of Reading in partial fulfilment for the degree of Ph.D. It is not possible to publish all the tables, but complete sets have been deposited with the original thesis in the libraries of the University of Reading and the Central Veterinary Laboratory, Weybridge.

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suspension was passed through sterile glass wool, left at room temperature overnight. The bacterial cells, collected by centrifugation, were washed three times, suspended in formol saline and kept at 4°C as stock suspensions. For agglutinating antigens, the stock suspensions were diluted to an opacity approximating Brown tube 1.

For the production of O antigens, the growth was suspended in sterile distilled water, passed through sterile glass wool and centrifuged once at 7500 r.p.m. for thirty minutes. The cells were resuspended in distilled water and then boiled under a reflux condenser for two hours. After cooling, the cells were centrifuged, resuspended in 0·4 per cent sterile saline and stored as stock suspensions at 4°C. For agglutinating antigens, the stock suspensions were diluted in 0·4 per cent saline to an opacity approximating Brown tube 1.

Boiled suspensions were also prepared by heating in N sterile saline in boiling water for various times, wa hing once in N saline and finally resuspending in N saline. Alcoholized suspensions were also prepared according to the method described by Blakemore and Gledhill (1946) by suspending the growth in absolute alcohol and heating to 56°C. for thirty minutes. The cells were collected by centrifugation and resuspended in N saline. All antigens were diluted to Brown tube 1 for agglutination tests.

For the purposes of this study, OH antigens are taken to denote suspensions in formol saline and O antigens as suspensions in distilled water boiled under a reflux condenser for two hours and resuspended in 0·4 per cent saline. Other antigens are described according to the methods of preparation.

*Preparation of antisera.* Rabbits were immunized by intravenous injections with stock suspensions of OH and O antigens (approximately 2 x Brown 10), seven injections being given twice weekly. The initial injections were 0·25 ml. and increased to 2·0 ml. for the last injection. Rabbits were bled before the injections and test bleedings made ten days after the last injection. If a satisfactory titre was shown, the rabbits were bled twice. The serum was collected and pooled, Seitz filtered and preserved with 0·5 per cent phenol and stored at 4°C. The sera produced against formolized suspensions are called OH and those produced against suspensions boiled under a reflux condenser for two hours are called O. Other sera are described according to the treatments given to the suspensions used for immunization.

*Agglutination test.* Where large numbers of agglutination tests with high titre sera had to be done, the technique recommended by Burrows, Mather, McGann and Wagner (1946) was used, the final dilutions ranging from 1:100 to 1:50,000. With other sera, serial two-fold dilutions were made starting at 1:20, to give dilutions ranging from 1:40 to 1:5120. Controls, consisting of antigen plus saline were always set up.

Tubes were routinely left in the 37°C incubator overnight and read after standing at room temperature for three hours. For comparative purposes in preliminary experiments, duplicate sets were also set up and incubated at 50°C. in a water bath and readings taken after three hours and again after having been left at room temperature overnight. The readings were interpreted as follows: ++++ = complete agglutination, clear supernatant; +++ = nearly complete agglutination, cloudy supernatant; ++ = marked deposit, supernatant opaque; + = slight

but definite deposit, supernatant opaque; — = no agglutination. The titre of the serum was taken as the highest dilution giving a + reading.

*Agglutinin-absorption.* Both OH and O suspensions were used for absorptions. OH sera were absorbed with the homologous O suspensions for the preparation of pure H sera which were then absorbed with OH suspensions for the study of the distribution of H antigens. O sera were absorbed with O suspensions for studying the O antigenic structure.

For the absorption of O sera and O antigens, O sera were diluted 1/40 and 6 ml. added to the centrifuged deposit of 6 ml. dense suspension of the O antigen, well mixed and left at 37°C. for three hours. The cells were then centrifuged off and the serum tested. It was found that one absorption was usually sufficient. For the absorption of OH sera and O antigens, OH sera were diluted 1/10 to 1/40, depending on the homologous titre and 40 ml. serum were added to the centrifuged deposit of 15 ml. of the homologous O antigen, left at 37°C. for three hours and the cells separated by centrifugation. In most cases, one absorption was sufficient. For the absorption of pure H sera and OH antigens both OH and pure H sera were diluted to approximately 1/64 of the homologous titre and 6 ml. were added to the deposited cells of 16 ml. of OH antigen, mixed and left at 37°C. for three hours, followed by centrifugation. Considerable difficulty was experienced in securing complete absorption and the process had to be repeated as many as four times or more. In addition, absorptions involving OH sera and boiled and alcoholized suspensions were also carried out. The same principles were followed except that absorption was allowed to proceed overnight at 37°C. followed by two hours at 4°C.

TABLE 1

THE EFFECT OF TEMPERATURE OF INCUBATION ON THE AGGLUTINATION TITRE

| *Serum* | *Antigen* | *Temperature of incubation* | *Titre* |
|---|---|---|---|
| Grant OH | Grant OH | 50°C | 1:20,000 |
| | Grant OH | 37°C | 1:50,000 |
| RBP11 OH | RBP 11 OH | 50°C | 1:10,000 |
| | RBP 11 OH | 37°C | 1:20,000 |
| HBC 1 OH | HBC 1 OH | 50°C | 1:10,000 |
| | HBC 1 OH | 37°C | 1:20,000 |

## RESULTS

### *Preliminary Experiments*

*Effect of temperature on the agglutination titre.* OH sera from three strains—HBC 1, RBP11 and Grant were used. Serial two-fold dilutions were set up in duplicate and equal volumes (0·5 ml.) of OH antigen added. After mixing, one set was left at 37°C. overnight followed by a few hours at room temperature; the second set was left at 50°C. for four hours followed by overnight incubation at room temperature. The results are shown in Table 1. Although

there was only one tube difference, the end point was easier to read in tubes incubated at 37°C.; moreover, because of the greater convenience of incubation at 37°C, this temperature was routinely adopted. Comparisons were also made with O sera and O antigens. As no significant differences in titre were observed, 37°C. was chosen for routine use.

*Comparison of readings after three and twenty-four hours.* Agglutination tests were set up using OH sera and OH antigens and readings taken after three and twenty-four hours at 37°C. with the results shown in Table 2. Although agglutination had occurred after three hours incubation, it was not complete until twenty-four hours. The end point was also easier to read after twenty-four hours.

TABLE 2

COMPARISON OF TITRES AFTER 3 AND 24 HOURS OF INCUBATION AT 37°C

| *Serum* | *Antigen* | *Titre after* | |
|---|---|---|---|
| | | *Three hours* | *Twenty-four hours* |
| 64/3 OH | 64/3 OH | 1280 | 2560 |
| DT 74 OH | DT 74 OH | 2560 | 2560 |
| Langford OH | Langford OH | 320 | 1280 |
| Langford 2 OH | Langford 2 OH | 320 | 1280 |

In order to determine whether this early agglutination was due to O or H antigen-antibody reactions, comparisons were made using pure H sera and OH antigens. Two representative results (Table 3) show that H agglutination does occur after three hours of incubation and is complete by twenty-four hours. No observations were made in the intervening period. In the case of agglutinations involving O sera and O antigens, visible agglutination took longer to occur, usually occurring after six hours. There was no difference in titres obtained after twenty-four hours incubation, unless otherwise stated.

TABLE 3

TITRES OBTAINED USING H SERA AND OH ANTIGEN AFTER 3 AND 24 HOURS OF INCUBATION

| *Serum* | *Antigen* | *Titre after* | |
|---|---|---|---|
| | | *Three hours* | *Twenty-four hours* |
| HBC 1 H | HBC 1 OH | 1:800 | 1:6400 |
| Grant H | Grant OH | 1:800 | 1:12,800 |

*Effect of boiling and alcohol.* Strain HBC 1 was grown on Thio blood agar for four days and the growth, suspended in N. saline, was divided into seven lots. Each batch was centrifuged at 7500 r.p.m. for thirty minutes and treated as follows: cells resuspended in N. saline—live saline suspension; cells suspended in formol saline—formolized suspension; cells suspended in N. saline immersed in boiling water for ten minutes, cooled and washed; cells suspended in N. saline, immersed in boiling water for thirty minutes, cooled and washed; cells suspended in absolute alcohol, heated to 56°C for thirty minutes, cooled and washed; cells suspended in N. saline and immersed in a boiling water bath for two hours; cells suspended in distilled water and boiled under a reflux condenser for two hours.

These suspensions were used for agglutination and agglutinin-absorption tests. The results (Table 4) show that alcohol destroys the agglutinability of the suspensions, but not their agglutinin-binding capacity. Similarly, boiling for ten minutes destroyed agglutinability which was partly restored after boiling for thirty minutes. In both cases agglutinins were absorbed by such boiled suspensions.

TABLE 4

THE EFFECT OF HEAT AND ALCOHOL ON THE AGGLUTINABILITY AND AGGLUTININ-BINDING CAPACITY

| *Serum* | *Absorbed* | *Antigen* | *Titre* |
|---|---|---|---|
| 1 OH | Not absorbed | Live saline suspension | 1:5000 |
| | | Formolized suspension (OH) | 1:5000 |
| | | Saline suspension boiled ten minutes | 0 |
| | | Saline suspension boiled thirty minutes | 1:200 |
| | | Saline suspension boiled two hours | 1:400 |
| | | Suspension boiled under reflux condenser for two hours (O) | 1:640 |
| | | Alcohol treated | 0 |
| ,, | Alcohol treated | Alcohol treated | 0 |
| | | Formolized (OH) | 0 |
| | | Boiled two hours | 0 |
| ,, | Formolized | Alcohol treated | 0 |
| | | Formolized (OH) | 0 |
| | | Boiled two hours | 0 |
| ,, | Boiled thirty minutes | Alcohol treated | 0 |
| | | Formolized (OH) | 0 |
| | | Boiled two hours | 0 |
| ,, | Boiled two hours | Formolized (OH) | 1:2560 |
| | | Boiled two hours | 0 |
| | Boiled under reflux condenser | Formolized (OH) | 1:5000 |
| | | Boiled under reflux condenser (O) | 0 |

All antigens and the serum HBC 1.

With suspensions boiled for two hours or boiled under a reflux condenser, titres against OH sera were very reduced. After absorption of OH Sera and O suspensions, titres against OH suspensions were practically the same as before absorption. It was found, by using other strains, that the results were more reproducible by boiling under a reflux condenser in the agglutinin-absorption tests and this was routinely used for preparing O suspensions. It was also found that boiling formolized suspensions gave irregular results.

The purity of the O sera was checked by the method of Burrows *et al.* (1946). O sera, absorbed with homologous O suspension, were tested against O and OH suspensions. In all cases agglutinins were completely absorbed, showing that, by boiling under a reflux condenser, antigenicity of the H antigen was completely destroyed.

*Analysis of the O Antigens*

The results of cross agglutination tests using six O sera and ten O antigens are given in Table 5 and those with OH sera and O antigens from the same strains in Table 6. The results (Table 5) showed the existence of two main groups, although cross agglutination to a minor degree occurred with some of the sera. The existence of two main O groups was also shown when agglutination tests were made using OH sera and O antigens, although again some slight degree of cross agglutination occurred. The results of absorption of O sera with O antigens showed that antigens of the homologous group completely removed all agglutinins whereas antigens of the heterologous group left agglutinins for homologous antigens. Occasionally, there was a reduction in titre, usually a one tube difference.

TABLE 5

AGGLUTINATION TESTS USING O SERA AND O ANTIGENS

| *O Sera** | *HBC1* | *ED49* | *Grant* | *156 AH77* | *5466* | *64/3* | *DT74* | *Langford* | *Langford 2* | *Bridge 1* |
|---|---|---|---|---|---|---|---|---|---|---|
| HBC 1 | 3200 | 6400 | 6400 | 3200 | 3200 | 0 | 100 | 100 | 100 | 0 |
| ED 49 | 640 | 1280 | 640 | 640 | 640 | 0 | 0 | 0 | 0 | 0 |
| 156AH77 | 2560 | 2560 | 640 | 1280 | 2560 | 0 | 0 | 0 | 0 | 0 |
| 64/3 | 0 | 0 | 0 | 0 | 80 | 2560 | 2560 | 2560 | 2560 | 2560 |
| Langford | 0 | 0 | 0 | 0 | 80 | 2560 | 2560 | 5120 | 5120 | 2560 |
| Bridge 1 | 0 | 0 | 0 | 80 | 80 | 2560 | 2560 | 1280 | 2560 | 2560 |

*Numbers denote reciprocal of highest dilution giving + reading.

On the basis of these results, two O groups were shown to exist. Group A was represented by strains HBC1, Grant, ED49, 156AH77 (bovine strains) and 5466 (sheep) and group B by strains 64/3, DT74, Bridge 1, (bovine), Langford and Langford 2 (sheep). Strains 156AH77 and 5466 were American strains; the remainder were isolated in this country.

Agglutination tests were also made using sera of each O group against a further 15 strains of *V. fetus*. Thirteen strains belonged to group A and two to group B. The distribution and origin of the strains are shown in Table 7. O antigens were also prepared from twelve non-pathogenic (catalase negative) vibrios and agglutination tests made against the six O sera of *V. fetus*. In no case did agglutination occur.

TABLE 6

AGGLUTINATION TESTS USING OH SERA AND O ANTIGENS

| *OH Sera** | *HBC1* | *ED49* | *Grant* | *156 AH77* | *5466* | *64/3* | *DT74* | *Langford* | *Langford 2* | *Bridge 1* |
|---|---|---|---|---|---|---|---|---|---|---|
| HBC 1 | 640 | 640 | 640 | 320 | 320 | 0 | 160 | 0 | 0 | 0 |
| ED 49 | 80 | 160 | 80 | 0 | 0 | 0 | 0 | 80 | 0 | 0 |
| Grant | 640 | 160 | 160 | 160 | 160 | 80 | 0 | 80 | 80 | 80 |
| 156AH77 | 1280 | 640 | 1280 | 1280 | 640 | 0 | 0 | 0 | 0 | 0 |
| 5466 | 320 | 640 | 80 | 160 | 320 | 0 | 160 | 160 | 0 | 0 |
| 64/3 | 0 | 160 | 0 | 0 | 0 | 5120 | 1280 | 1280 | 2560 | 5120 |
| DT74 | 0 | 160 | 0 | 0 | 160 | 1280 | 160 | 640 | 640 | 320 |
| Langford | 0 | 0 | 0 | 0 | 0 | 2560 | 320 | 1280 | 1280 | 5120 |
| Langford 2 | 0 | 160 | 0 | 80 | 80 | 2560 | 2560 | 5120 | 5120 | 5120 |
| Bridge 1 | 0 | 0 | 0 | 0 | 0 | 2560 | 1280 | 1280 | 1280 | 2560 |

*Numbers denote reciprocal of highest dilution giving + reading.

TABLE 7

DISTRIBUTION OF STRAINS

| *O group* | *No. of strains* | *Origin* |
|---|---|---|
| A | 18 | 12 bovine<br>6 sheep |
| B | 7 | 5 bovine<br>2 sheep |

*Analysis of the H Antigens*

OH sera were prepared against three strains of *V. fetus* and agglutination tests were made using these three sera and OH antigens prepared from 60 strains of *V. fetus*—eight of sheep and fifty-two of bovine origin. Twelve strains were received from America, three from Denmark and the remainder were isolated in this country. Considerable cross agglutination occurred, with titres varying from 1/1000 to 1/20,000. However, three strains were agglutinated either to a low titre (1/200), or not at all.

Ten strains were chosen for the preparation of fresh OH sera and for a more detailed study involving the production of pure H sera (by absorption with homologous O antigens) and for absorption of pure H sera with OH suspensions. The results, using pure H sera, obtained by absorption, and OH antigens, are given in Table 8. In addition, agglutination tests were also made using H sera against other OH antigens. Representative results obtained with serum Grant are shown in Table 9.

TABLE 8

AGGLUTINATION TESTS. PURE H SERA AND OH ANTIGENS

| *Antigens* | *Sera* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *HBC1* | *Grant* | *ED49* | *156AH77* | *5466* | *64/3* | *DT74* | *Langford* | *Langford 2* | *Bridge 1* |
| *HBC 1* | **10000** | 20000 | 6400 | 5000 | 2560 | 320 | 640 | 2560 | 2560 | 1280 |
| Grant | 10000 | **50000** | 12800 | 10000 | 20000 | 5120 | 1280 | 5120 | 1280 | 5120 |
| ED49 | 5000 | 1280 | **12800** | 5000 | 320 | 320 | 320 | 5120 | 1280 | 1280 |
| 156AH77 | 2500 | 320 | 800 | **1280** | 320 | 0 | 40 | 160 | 160 | 640 |
| 5466 | 2500 | 20000 | 6400 | 5000 | **20480** | 640 | 1280 | 1280 | 640 | 2560 |
| 64/3 | 160 | 320 | 1600 | 160 | 320 | **2560** | 80 | 160 | 320 | 1280 |
| DT74 | 5000 | 20000 | 1600 | 5000 | 5120 | 2560 | **5000** | 5120 | 640 | 2560 |
| Langford | 320 | 320 | 400 | 160 | 320 | 1280 | 80 | **1280** | 640 | 1280 |
| Langford 2 | 1280 | 1280 | 3200 | 640 | 2560 | 1280 | 640 | 2560 | **2560** | 5120 |
| Bridge 1 | 80 | 160 | 400 | 80 | 320 | 1280 | 0 | 320 | 320 | **640** |

The results shown in Table 8 revealed a considerable cross agglutination between pure H sera and OH antigens although quantitative differences were shown. Certain strains e.g. Bridge 1, Langford, 64/3 and 156AH77 were agglutinated to a comparatively low titre against some sera whereas the sera of these strains agglutinated all strains to a high titre. After absorption of OH sera with homologous O antigen for the preparation of pure H sera, there was often a reduction in titre but it was not large since normally there was only a difference of one tube. On two occasions, whereas the unabsorbed (OH) sera did not agglutinate a particular strain, the absorbed (H) sera did. These tests have been repeated with similar results.

Other strains examined (Table 9) were also agglutinated by pure H serum against strain Grant. Results with other H sera (not tabulated) also showed considerable cross agglutination. Formolized suspensions of catalase negative (non-pathogenic) vibrios were not agglutinated by either OH or H sera prepared against ten strains of *V. fetus*.

Agglutinin-absorption tests were made using the pure H sera listed in Table 8 and the OH suspensions. Each serum was absorbed with each of the 10 suspensions and agglutination tests made against the absorbing and homologous antigens. Representative results obtained with four sera are shown in Table 10.

The results of absorption tests revealed the existence of type specific H components in addition to the common H component. In the case of four strains, viz: 64/3, Langford, Langford 2 and Bridge 1, it was found that the type specific components were

identical. However, with the remaining strains, it was found that, whereas agglutinins against the absorbing strain had been removed, agglutinins against the homologous strain still remained, often to a high titre. These sera had been absorbed on four successive occasions with the absorbing strain, the titres after the third and fourth absorptions being practically the same. In all cases, absorption of a serum with homologous suspension removed all agglutinins and absorption was usually complete after two successive absorptions.

TABLE 9

AGGLUTINATION TEST USING GRANT H SERUM AND OH ANTIGENS

| *Antigens (a)* | *Titre** |
|---|---|
| RBP 11 | 12,800 (20,000) |
| BF 78 | 12,800 (10,000) |
| (D) Aaltje | 800 ( 5,000) |
| 155 | 6,400 (20,000) |
| 1980 | 1,600 ( 5,000) |
| Hume | 12,800 (50,000) |
| Bridge 2 | 1,600 (20,000) |
| (A) 35-299 | 1,600 ( 1,600) |
| (A) 169 T23 | 0 ( 500) |
| (A) 5623 | 400 (10,000) |
| (A) 9DLDU | 0 ( 1,000) |
| (AS) 5090 | 3,200 (10,000) |
| (A) 4404 | 400 (10,000) |
| (A) 200 Mum | 3,200 (10,000) |
| (A) 6RCM | 200 ( 1,000) |
| Barrett | 3,200 (10,000) |
| MS 76 | 6,400 (10,000) |
| (S) 387 | 10,240 (20,000) |
| (S) 396 | 5,120 (10,000) |
| (S) 424 | 5,120 (10,000) |
| (S) 835 | 5,120 (10,000) |
| (S) 845 | 5,120 (10,000) |

(a) D = Dutch strain
A = American strain
S = sheep strain
All others bovine strains

*Numbers denote reciprocal of highest dilution giving reading. Numbers in brackets are titres against unabsorbed (OH) sera.

In order to determine further the distribution of the major H components, attempts were made to produce sera specific for the major components by absorption with an antigen and testing this absorbed serum against all ten antigens. From these results (Table 11) it is apparent that strains HBC 1, Grant, ED49, 5466 and DT74 possess a common type specific antigen but, from the results of absorptions shown in Table 10 they also possess strain specific antigens. Strains 64/3, Bridge 1, Langford and Langford 2 possess the same type specific antigens, but do not possess additional strain

TABLE 10

ABSORPTION OF H SERA AND OH SUSPENSIONS

| *H Serum* | *Absorbed* | *O-H Antigen* | *Titre* | |
|---|---|---|---|---|
| | | | *Before absorption* | *After absorption* |
| HBC1 | HBC1 | HBC1 | 10000 | 0 |
| | Grant | Grant | 10000 | 0 |
| | | HBC1 | 10000 | 2500 |
| | ED49 | ED49 | 5000 | 0 |
| | | HBC1 | 10000 | 5000 |
| | 64/3 | 64/3 | 160 | 0 |
| | | HBC1 | 10000 | 2500 |
| | DT74 | DT74 | 5000 | 0 |
| | | HBC1 | 10000 | 5000 |
| | Langford | Langford | 320 | 0 |
| | | HBC1 | 10000 | 10000 |
| | Langford 2 | Langford 2 | 1280 | 0 |
| | | HBC1 | 10000 | 10000 |
| | Bridge 1 | Bridge 1 | 80 | 0 |
| | | HBC1 | 10000 | 2500 |
| | 156AH77 | 156AH77 | 2500 | 0 |
| | | HBC1 | 10000 | 2500 |
| | 5466 | 5466 | 2500 | 0 |
| | | HBC1 | 10000 | 5000 |
| Langford | HBC1 | HBC1 | 2560 | 0 |
| | | Langford | 1280 | 1280 |
| | Grant | Grant | 5120 | 0 |
| | | Langford | 1280 | 640 |
| | ED49 | ED49 | 5120 | 0 |
| | | Langford | 1280 | 640 |
| | 64/3 | 64/3 | 320 | 0 |
| | | Langford | 1280 | 0 |
| | DT74 | DT74 | 5120 | 0 |
| | | Langford | 1280 | 1280 |
| | Langford | Langford | 1280 | 0 |
| | Langford 2 | Langford 2 | 2560 | 0 |
| | | Langford | 1280 | 0 |
| Langford | Bridge 1 | Bridge 1 | 320 | 0 |
| | | Langford | 1280 | 0 |
| | 156AH77 | 156AH77 | 160 | 0 |
| | | Langford | 1280 | 320 |
| | 5466 | 5466 | 1280 | 0 |
| | | Langford | 1280 | 640 |

*Table* 10 *continued*

| *Serum* | *Absorbed* | *O-H Antigen* | *Titre* *Before absorption* | *After absorption* |
|---|---|---|---|---|
| Langford 2 | HBC1 | HBC1 | 2560 | 0 |
| | | Langford 2 | 2560 | 1280 |
| | Grant | Grant | 1280 | 0 |
| | | Langford 2 | 2560 | 1280 |
| | ED49 | ED49 | 1280 | 0 |
| | | Langford 2 | 2560 | 1280 |
| | 64/3 | 64/3 | 320 | 0 |
| | | Langford 2 | 2560 | 0 |
| | DT74 | DT74 | 640 | 0 |
| | | Langford 2 | 2560 | 2560 |
| | Langford | Langford | 640 | 0 |
| | | Langford 2 | 2560 | 0 |
| | Langford 2 | Langford 2 | 2560 | 0 |
| | Bridge 1 | Bridge 1 | 320 | 0 |
| | | Langford 2 | 2560 | 0 |
| | 156AH77 | 156AH77 | 160 | 0 |
| | | Langford 2 | 2560 | 640 |
| | 5466 | 5466 | 640 | 80 |
| | | Langford 2 | 2560 | 2560 |
| 64/3 | HBC1 | HBC1 | 320 | 0 |
| | | 64/3 | 2560 | 640 |
| | Grant | Grant | 5120 | 0 |
| | | 64/3 | 2560 | 1280 |
| | ED49 | ED49 | 320 | 0 |
| | | 64/3 | 2560 | 1280 |
| | 64/3 | 64/3 | 2560 | 0 |
| | DT74 | DT74 | 2560 | 0 |
| | | 64/3 | 2560 | 320 |
| | Langford | Langford | 1280 | 0 |
| | | 64/3 | 2560 | 0 |
| | Langford 2 | Langford 2 | 1280 | 0 |
| | | 64/3 | 2560 | 0 |
| | Bridge 1 | Bridge 1 | 1280 | 0 |
| | | 64/3 | 2560 | 0 |
| | 156AH77 | 156AH77 | 0 | 0 |
| | | 64/3 | 2560 | 1280 |

specific antigens, Strains 156AH77 contains its own type specific antigen, but a serum against this component was not prepared.

The analysis was not extended to further strains nor to include all minor antigens in the strains studied. In the light of the present studies, antigenic formulae proposed for the strains are given in Table 12. All the strains possess a common H component 1. Five possess the type specific component 2, one the component 3 and four the component 4. In addition, five possess strain specific components 5 to 9; four did not possess a strain specific component and, in the case of one strain (156AH77) it was not determined. There was no connexion between the O groups and the type specific H components.

TABLE 11

| *Serum* | *Absorbed with* | *Antigens* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *HBC1* | *Grant* | *ED49* | *156AH77* | *5466* | *64/3* | *DT74* | *Langford* | *Langford 2* | *Bridge 1* |
| HBC1 | Langford | 4800 | 2400 | 1200 | 0 | 600 | 0 | 0 | 0 | 0 | 0 |
| ED49 | 64/3 | 1280 | 2560 | 2560 | 0 | 640 | 0 | 160 | 0 | 0 | 0 |
| DT74 | 64/3 | 320 | 2560 | 1280 | 0 | 320 | 0 | 1280 | 0 | 0 | 0 |
| Langford | HBC 1 | 0 | 0 | 0 | 0 | 0 | 1280 | 0 | 320 | 640 | 640 |
| 64/3 | HBC 1 | 0 | 0 | 0 | 0 | 0 | 2560 | 0 | 1280 | 1280 | 2560 |

TABLE 12

ANTIGENIC STRUCTURE

| *Strain* | *O Group* | *H components* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Common* | *Type specific* | | | *Strain specific* | | | | | |
| | | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | |
| HBC 1 | A | + | + | | | + | | | | | |
| Grant | A | + | + | | | | + | | | | |
| ED 49 | A | + | + | | | | | + | | | |
| 156AH77 | A | + | | + | | | | | | | |
| 5466 | A | + | + | | | | | | + | | |
| 64/3 | B | + | | | + | | | | | | |
| DT 74 | B | + | + | | | | | | | + | |
| Langford | B | + | | | + | | | | | | |
| Langford 2 | B | + | | | + | | | | | | |
| Bridge 1 | B | + | | | + | | | | | | |

## DISCUSSION

The results reported in the present work allow an analysis to be made of the distribution of the H and O antigenic components of *V. fetus* and their relationship to the non-pathogenic, catalase negative vibrios. Two O groups were found, based on cross agglutination tests using both O and OH sera against O antigens and by agglut-

inin-absorption tests using O sera and O antigens. Both sheep and bovine strains of *V. fetus* were found in both the O groups. Smith and Orcutt (1927) studying the antigenic relationship of *V. fetus* and vibrios isolated from the intestines of calves found that *V. fetus* and the calf vibrios belonged to two distinct O groups although sharing common flagellar antigens. All the strains of *V. fetus* studied by these workers belonged, therefore, to one O group.

Price, Poelma and Faber (1955) also studied extensively the O antigenic structure of *V. fetus* and found five O groups which they designated I to V. However, O groups I, II and III shared a common component A and groups I and III shared a common component C. Group V was completely unrelated and contained component Y. O suspensions were prepared by these workers by immersing saline suspensions in a boiling water bath for two hours. Gardner and Venkatraman (1935) found that immersing suspensions of *V. cholerae* in boiling water to destroy the H antigen resulted in the appearance of a new O antigen common to a variety of heated vibrios. Burrows *et al.* (1946), found that immersing suspensions of *V. cholerae* in boiling water did not completely destroy the antigenicity of the flagellar antigen and that when such sera were absorbed with boiled suspensions, traces of H antibody were still left. Antigenicity of the H antigen was completely destroyed only by boiling under a reflux condenser.

In the present work, it was found that immersing saline suspensions of *V. fetus* in boiling water for thirty minutes considerably reduced the agglutinability of the suspension but did not destroy the agglutinin-binding capacity. OH sera absorbed with such boiled suspensions did not agglutinate OH suspensions. It was also found, by agglutinin-absorption experiments, that even immersing in boiling water for two hours did not always completely destroy agglutinin-binding capacity. Boiling under a reflux condenser for two hours was found to be the procedure that gave consistent results.

Blakemore and Gledhill (1946) prepared O suspensions of *V. fetus* by heating them at 56°C in absolute alcohol for thirty minutes and concluded that the O antigen was type specific in sheep strains. In the present work it was found that heating suspensions in absolute alcohol rendered the strains completely inagglutinable, but did not destroy the agglutinin-binding capacity. Gardner and Venkatraman (1935) also found that alcohol rendered the cholera vibrios inagglutinable and that such suspensions were unsuitable for the preparation of O sera.

Juhler (1955) prepared O suspensions by boiling formolized suspensions for two hours and sera were prepared against six such suspensions. Four O groups were found to occur. The first group consisted of three strains which also possessed common H antigens. Groups 2, 3 and 4 were represented by one strain each and each one also possessed distinct H antigens. The titres were generally in the region of 1:320. The question still arises as whether the

antigenicity of the flagellar antigen had been completely destroyed by the treatment used.

Smith and Taylor (1919) and Smith and Orcutt (1927) also showed that bovine strains of *V. fetus* shared a common H antigen in addition to possessing a type specific H antigen. This work has been criticized as all the strains were isolated from one large herd and were not, therefore, completely representative. Subsequent reports on the serology of *V. fetus* have in the main included only direct cross agglutination tests without recourse to agglutinin-absorption experiments. In no case have attempts been made to prepare pure H sera by absorption with homologous O antigens.

Most workers have found that there is a considerable degree of cross agglutination between strains of *V. fetus* although it has occasionally been found that a few strains are quite distinct from those comprising the main group and from each other (Levi, 1950; Marsh and Firehammer, 1953; Price, Poelma and Faber, 1953; Gallut, 1952; Juhler, 1955; Wiidik and Hlidar, 1955). Price *et al.* (1953) who studied thirty strains, found that the differences in the H antigens, which were quantitative rather than qualitative, were not sufficiently clear cut to allow separation into groups. It was also noted that some strains were not agglutinated by all the sera although sera against such strains agglutinated all the suspensions. This occurred in the present studies e.g. serum DT74 did not agglutinate antigen Bridge 1 but serum Bridge 1 agglutinated antigen DT74 (Table 8). These results might represent quantitative rather than qualitative differences.

Biberstein (1956), on the basis of agglutinin-absorption tests using OH sera and OH antigens, postulated the existence of a large number of haptenes which were held to be responsible for the wide degree of serological reactions observed. Similar observations were made by Gallut (1952). In the present work, agglutination and agglutinin-absorption tests using H sera and OH suspensions showed that strains possess a common H antigen. In addition, type specific H components were also found to occur; these were occasionally shared by a number of strains. Some strains also possessed strain specific components. Four strains—two sheep and two bovine strains—were shown to possess the same type specific H component, but did not have strain specific components. Five strains sharing the same type specific component 2 also possessed strain specific components. Type specific H components were not specific for any O group.

Burrows *et al.* (1946) noted that O agglutination of the cholera vibrio occurred after two hours' incubation, but H agglutination was not complete until twelve to eighteen hours. They also noted that it was not possible to differentiate between O and H agglutination. Price *et al.* (1955) stated that the O agglutination was more granular and was complete in twenty-four hours whereas Wiidik and Hlidar (1955) stated that O agglutination could be read after two hours incubation and H agglutination only after twenty-four

hours incubation. Plastridge, Williams, Easterbrooks, Walker and Beccia (1951) observed that O agglutination was complete after twenty-four hours and H agglutination after forty-eight to seventy-two hours. In the present work, using pure H sera against OH suspensions, visible agglutination occurred after three hours' incubation and was complete after twenty-four hours. In the case of O sera and O antigens, visible agglutination did not occur until six hours' incubation and was complete in twenty-four hours. In either case, there was no difference between results obtained after twenty-four and forty-eight hours.

Catalase negative, non pathogenic vibrios were not agglutinated by either O or OH sera prepared against any of the strains studied thus confirming the work of Price *et al.* (1953) and Biberstein (1956); Bond (1957) suggested that the catalase negative vibrios were, in effect, mucoid variants of *V. fetus*. These mucoid variants were, however, non-motile whereas the catalase negative vibrios were actively motile (Thouvenot and Florent, 1954; personal observations). Furthermore, it was found by Bond (1957) that the mucoid forms were not agglutinated by sera produced against either smooth or rough strains of *V. fetus*; neither were smooth and rough antigens agglutinated by sera produced against the mucoid variants. There was, therefore, no antigenic relationship at all between the smooth and rough forms of *V. fetus* and mucoid forms. If they were variants of the same species, it would have been expected that they would at least share the rough antigens as is the case in the salmonellas. The fact that these catalase negative vibrios differ from *V. fetus* biochemically and serologically, as well as in pathogenicity (Frank, Bryner and Caruthers, 1956), supports the view of Thouvenot and Florent (1954) that they are, in effect, two different species.

## CONCLUSIONS

By immunizing rabbits with suspensions of *V. fetus* that had been boiled under a reflux condenser for 2 hours and using such sera for agglutination and agglutinin-absorption tests, two O groups were detected. Both sheep and bovine strains were found in each group.

By absorbing OH sera with homologous O suspensions, sera containing only H antibodies were obtained and these were used for agglutination and agglutinin-absorption tests. Nearly all strains contained a common H component: furthermore, additional type specific H components common to a few strains as well as strain specific H components occurred.

Neither the O nor the H sera agglutinated non-pathogenic, catalase negative vibrios.

## ACKNOWLEDGMENTS

The author wishes to thank Dr. A. T. R. Mattick of the National Institute for Research in Dairying, Shinfield for allowing facilities for

this work and for his helpful criticisms and suggestions at all times. Thanks are also due to various colleagues for sending strains of *V. fetus*.

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[*Received for publication, July 3rd*, 1958]

# GOAT POX IN IRAN

## Serial Passage in Goats and the Developing Egg, and Relationship with Sheep Pox

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### INTRODUCTION

Goat pox, first described by Hansen in Norway in 1879, is a common disease among goats in the Middle-Eastern countries. Although the disease is usually comparatively benign, it is sometimes characterized by severe symptoms and constitutional disturbances which may result in death. The virus can be transmitted to the goat by a variety of routes.

Thus Melanidi and Tzortzaki (1937) found that the inoculation of goat virus by the intradermal, subcutaneous, intravenous and intraperitoneal routes caused generalized lesions. Intratracheal inoculation induced a rise in temperature followed by generalization, while intracerebral injection caused an encephalitis and death. Kerato-conjunctivitis followed the inoculation of the virus into the cornea. Goats were not susceptible by the mouth.

A number of workers have studied the relationship between sheep pox and goat pox viruses in an attempt to produce a single vaccine which would protect sheep and goats against both diseases. Grimpret (1938), Kolayli, Marvidis and Ilhami (1933), and Slagsvold (1942) claim that they infected sheep with goat pox virus. In addition, some of these workers state that they could immunize sheep by using a vaccine prepared with the goat pox virus. Bennett, Horgan and Mansur (1944) and Lall, Singh and Singh (1947) failed, however, to confim these findings.

During the past two years we have undertaken an investigation on the transmission and cultivation of the virus, and on cross-immunity experiments with sheep pox and goat pox viruses respectively.

### NATURAL DISEASE

The number of infected animals in a flock may vary from 5 to 100 per cent. In the acute stage, the lesions may appear over the whole of the body but particularly around the mouth and eyes, on the udder and teats, on the scrotum and on the inner side of the thighs. The disease follows the same stages as other members of the pox group. After a period of incubation of 14 to 17 days (Kolayli, *et al.*, 1933) the temperature rises to 40 to 41°C and the animal suffers from anorexia. A few days later, small greyish-red papules are formed which are later converted into nodules about the size of a lentil; these nodules, which contain a little clear lymph,

form the vesicles. The content of the vesicles becomes turbid, the surface becomes necrotic, and a yellowish purulent liquid which exudes dries into scabs and crusts. Recovery takes place in 25 to 30 days. In severe cases common symptoms are respiratory tract disturbances, enlargement of the udder and abortion. The horse, donkey, calf, sheep, pig, dog, rabbit and birds never contract the disease and the authors have failed to infect sheep by co-habitation with diseased goats. In experimental infection of goats the incubation period is reduced to 3 to 5 days.

In our experience, sheep pox virus rarely causes a typical local reaction in the goat.

## EXPERIMENTAL DISEASE

### *Production of Vaccine in Goats*

The method described in this paper follows closely that employed for the production of sheep pox and is based on the classical method of Borrel. A field virus from Iranian goats was inoculated into goats by the intradermal and subcutaneous routes. After four serial passages, the virus was propagated satisfactorily in the subcutaneous tissues producing a widespread oedematous reaction similar to that described by Borrel for sheep pox.

For the preparation of the vaccine, goats weighing 30 to 35 kg. are injected on both sides of the thorax. Temperatures are recorded daily. A rise in temperature occurs between the third and fourth day and lesions develop on the skin after 5 to 6 days. Goats showing fever and a local reaction are killed between the seventh and ninth days.

The hair on the underside of the trunk is clipped and the borders of each side cauterized. The whole surface is then disinfected with tincture of iodine and an incision is made through the cauterized parts. The skin together with adjacent tissues is lifted and held by 4 sterile hooks. The exudate and the oedematous tissue are harvested and ground in a Latapie blender.

Each goat yields approximately 800 g. of infected tissues and 100 ml. of liquid material. After checking for sterility the material is stored at —30°C until used. The virus does not lose its activity under these conditions. The local reaction to vaccination is not severe and goats receiving the material are not capable of infecting healthy goats placed in contact with them.

### *Culture of the Virus in the Developing Egg*

White Leghorn eggs incubated for 12 days are drilled according to the standard technique for chorio-allantoic inoculation. The inoculum (0·2 ml.) treated with 400 i.u. penicillin and 2 mg. streptomycin is deposited on the chorio-allantoic membrane and the hole in the shell sealed with Scotch tape. Ten eggs are used for each passage. After 3 to 4 days eggs with live embryos are put at

—20°C for 20 minutes. The chorio-allantoic membrane is harvested aseptically and checked for opaque spots. These lesions were detected in all passage material from the second to the twelfth. Infected membranes are ground in a sterile mortar and divided into two parts. One part is used for the next passage and the other lyophilized in an Edwards's apparatus and kept at low temperature.

The fourth and eighth egg passages were inoculated subcutaneously into two goats (70, and 79) respectively. Both animals showed a slight local reaction about the size of a hazel-nut.

*Immunity Induced by Vaccination*

Both sheep and goats have been used in these trials. The preparation of the goat vaccines has been described above. Some batches were added to aluminium gel and others were not. The sheep pox vaccine was absorbed on aluminium hydroxide and its preparation has been described elsewhere (Rafyi and Mir-Chamsy, 1956). For challenging the immunity, the virulent sheep pox virus was the 3rd passage of the Delbosc strain with a titre of 1/1,000,000. The goat pox was the 6th passage of an Iranian virus (Gorgan strain) with a titre of about 1/200,000. Vaccinated animals were challenged intradermally with 0·5 ml. of undiluted virus and controls with the same dose of a 1/200 dilution.

As there is a difference in susceptibility of some of the Iranian breeds to the pox viruses, four types of experiment were carried out. The technique was similar, but the breed of animals and the batch of vaccine were varied because of differences in the degree of susceptibility (Delpy and Rafyi, 1951). In these experiments 100 goats and sheep were divided into two groups. The first group was vaccinated subcutaneously with 0·5 ml. of goat pox vaccine and the second group with 0·5 ml. of the sheep pox vaccine by the same route.

*Vaccination of Goats.* Ten goats were vaccinated with absorbed goat pox vaccine and ten with sheep pox vaccine. Six controls were not vaccinated. Fifteen days later all the animals were challenged with goat virus with the results shown in Table 1. The animals protected with goat vaccine were completely immune but those which had received sheep vaccine showed a rise in temperature and a typical local reaction. All the controls reacted. It would appear, therefore, that goats vaccinated with the sheep pox vaccine are not protected against a challenge dose of the goat pox virus and that sheep pox vaccine cannot be used in the control of goat pox. The experiment was repeated on 25 goats and 4 sheep, but on this occasion the challenge dose was sheep pox virus. The results are also set out in Table 1.

In these experiments with goat pox vaccine no symptoms or lesions were observed in the ten vaccinated and three control goats; the four control sheep showed a typical pox reaction. Fifteen days later, the 13 goats were tested with goat pox virus; the ten vaccinated

goats did not react, whereas the controls showed a local reaction and hyperthermia.

In the case of the group vaccinated with sheep pox all the vaccinated and control goats failed to react, but the control sheep showed a mild but typical response. The ten vaccinated and two control goats were rechallenged with the goat pox virus and all 12 animals showed a local reaction and a rise in temperature.

TABLE 1

VACCINATION EXPERIMENTS IN GOATS

| *Vaccine* | *No. of animals* | *1st challenge* | | *2nd challenge* | |
|---|---|---|---|---|---|
| | | *Material* | *Result* | *Material* | *Result* |
| Goat pox + gel .. | 10 G | GPV und. | 0/10 | | |
| Sheep pox + gel .. | 10 G | GPV und. | 10/10 | | |
| — | 6 G | GPV 1/200 | 6/6 | | |
| Goat pox + gel .. | 10 G | SPV und. | 0/10 | GPV und. | 0/10 |
| Sheep pox + gel .. | 10 G | SPV und. | 0/10 | GPV und. | 10/10 |
| — | 5 G | SPV 1/200 | 0/5 | GPV 1/200 | 5/5 |
| — | 4 S | SPV 1/200 | 4/4 | — | |
| Goat pox without gel .. | 10 G | SPV und. | 0/10 | GPV und. | 0/10 |
| — | 2 G | — | | GPV 1/200 | 2/2 |
| — | 2 S | SPV 1/200 | 2/2 | — | |

G=goat S=sheep und.=undiluted
GPV=goat pox virus SPV=sheep pox virus

In addition to goat vaccine with aluminium gel a test was made with vaccine without gel. Ten goats were injected with vaccine. They were tested with sheep pox virus together with 2 control goats and 2 control sheep. The sheep reacted normally but all the goats remained negative. Fourteen days later all the goats were re-inoculated with goat virus. All the vaccinated animals were immune: the two controls reacted.

*Vaccination of Sheep.* Ten sheep were vaccinated with goat pox vaccine and ten with sheep pox vaccine. Fifteen days later they were challenged with goat pox virus. In the first group, there was no response in the vaccinated animals, but two control goats reacted. The vaccinated sheep with two control sheep were subsequently challenged with sheep pox virus: only the controls reacted.

TABLE 2

VACCINATION EXPERIMENTS IN SHEEP

| *Vaccine* | *No. of animals* | *1st challenge* | | *2nd challenge* | |
|---|---|---|---|---|---|
| | | *Material* | *Result* | *Material* | *Result* |
| Goat pox + gel .. | 10 S | GPV und. | 0/10 | SPV und. | 0/10 |
| Sheep pox + gel .. | 10 S | GPV und. | 0/10 | SPV und. | 0/10 |
| — | 4 G | GPV 1/200 | 4/4 | — | |
| — | 4 S | — | | SPV 1/200 | 4/4 |
| Goat pox + gel .. | 10 S | SPV und. | 0/10 | | |
| Sheep pox + gel .. | 10 S | SPV und. | 0/10 | | |
| — | 5 S | SPV 1/200 | 4/5 | | |
| Goat pox without gel .. | 10 S | SPV und. | 0/10 | | |
| — | 3 S | SPV 1/200 | 3/3 | | |

G=goat S=sheep und.=undiluted
GPV=goat pox virus SPV=sheep pox virus

TABLE 3

VACCINATION EXPERIMENT EGG VIRUS IN GOATS

| *No. of animal* | *Vaccination* | | | *Challenge* | | |
|---|---|---|---|---|---|---|
| | *Date* | *Material* | *Local reaction* | *Date* | *Material* | *Local reaction* |
| 70 | 30/3/57 | 4th passage | + | 14/4/57 | Goat virus undiluted | 0 |
| 79 | 13/6/57 | 8th passage | + | 28/6/57 | | 0 |
| 72 | | — | | 14/4/57 | Goat virus 1/200 | ++ |
| 80 | | — | | 28/6/57 | | ++ |

0=no reaction +=reaction size of hazel nut
++=reaction size of walnut with scabs

In the second group, there was no reaction in the vaccinated sheep to goat pox whereas two control goats reacted. After a second challenge with sheep pox the vaccinated animals were unaffected but two control sheep reacted (Table 2). These experiments would seem to establish the ability of goat pox vaccine to induce a solid immunity in sheep.

In the next trials 10 goats were vaccinated with goat pox and

10 with sheep pox. They were then tested for their resistance to sheep pox virus. All 20 sheep were immune whereas 4 out of 5 controls reacted (Table 2). Goat pox vaccine without aluminium gel gave complete protection to 10 sheep against sheep pox virus (Table 2).

*Vaccination with egg-adapted virus.* Two goats which had been inoculated, one with 4th passage egg virus and one with virus in the 8th passage gave slight local reactions. When tested 15 days later, they showed no reaction, whereas two controls developed a rise in temperature and local reactions (Table 3).

## DISCUSSION

Sheep pox and goat pox are two of the important virus diseases in Iran.

Various strains of sheep pox virus have been investigated for vaccine production in Iran since 1936. Freeze-dried virus adsorbed on anthrax spores (Delpy, Rafyi and Mir-Chamsy, 1951) was formerly used for mass immunization of sheep in this Institute. It was not possible, however, to produce a special vaccine for the control of goat pox until 1957 although veterinarians and farmers have sometimes used the combined vaccine in goats to protect them against anthrax without having any intention of immunizing them against goat pox. There has been a considerable difference of opinion about the efficacy of this vaccine in protecting goats against goat pox.

During the past two years large amounts of goat pox vaccine have been prepared and fairly extensive experiments have been completed. According to our findings, although goat pox virus does not produce a large reaction in sheep, it immunizes them against sheep pox. The duration of the immunity is at present unknown. On the other hand goats injected with virulent sheep pox virus or with sheep pox vaccine fail to react and are subsequently fully susceptible to goat pox.

The advantages of goat pox vaccine as produced by the methods outlined lies in its value as an efficient prophylactic not only against goat pox but also to protect sheep against sheep pox. It is emphasised that in order to immunise goats this special goat pox vaccine must be used.

## CONCLUSIONS

Goat pox virus can be easily isolated from naturally infected animals. It maintains its virulence after successive passages in susceptible goats.

The virus can be propagated in the developing hen's egg.

Sheep pox vaccine does not protect goats against goat pox: goat pox vaccine, on the other hand, affords a solid protection against both goat pox and sheep pox.

Egg-adapted goat pox virus can be used as a prophylactic.

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[*Received for publication, May 20th*, 1958]

# MORTALITY OF RABBITS INOCULATED WITH LAPINISED RINDERPEST VIRUS

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## INTRODUCTION

The strains of lapinised rinderpest virus in common usage are substrains of the Nakamura III strain (Nakamura, Wagatuma and Fukusho, 1938). In the earlier passages, the mortality in inoculated rabbits was low, but after the 600th passage more than 80 per cent of the rabbits died (Cheng and Fischmann, 1949). Later, Nakamura (1950) noted a range of 20 to 80 per cent in the mortality rates induced by different substrains of the virus. The Kabete substrain was initiated in 1949 for the production of an attenuated vaccine (Brotherston, 1951). The case mortality rate in rabbits induced by the Kabete substrain and its relationship with the amount of virus administered are herein recorded.

## MATERIALS AND METHODS

*Virus.* Stock aliquots from the 805th rabbit passage of the Nakamura III strain were prepared as 20 per cent infected rabbit tissue suspensions which were shell frozen in dry-ice and alcohol and dried under vacuum. The ampoules were stored at minus 20°C. until required. The virus was reconstituted with nutrient broth to give a $10^{-1}$ dilution per gram of wet tissue and thereafter serial tenfold dilutions were prepared in nutrient broth. Rabbits were inoculated intravenously with 1·0 ml. of the appropriate dilution of virus. The number of $ID_{50}$ inoculated was confirmed simultaneously by titrating the virus in rabbits, using four rabbits per dilution.

*Rabbits.* The rabbits were a heterologous mixture derived from the Albino, Rex, Chinchilla and Belgian hare breeds. They were used when 4 to 6 months old and weighed approximately 1·5 kg.

*Analysis.* Eight replicate experiments were made. The influence of the amount of virus administered on the case mortality was determined by comparing the 50 per cent death times and the Gompertz functions (Gompertz, 1825) associated with the high and low inocula. The 50 per cent death time is the period in which 50 per cent of the inoculated rabbits die. The Gompertz function is a straight line relating the logarithm of the rate of mortality to the post inoculation interval. The functions were derived by constructing life tables and calculating the rates of mortality at different times from the number of deaths occurring over a given interval. The significance of the regression lines was tested by the Analysis of Variance. The difference between the slopes of the Gompertz functions was tested for significance by Student's t test.

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## RESULTS

The morbidity rate and case mortality rate in rabbits inoculated intravenously with lapinised rinderpest virus were 100 and $95{\cdot}96 \pm 2{\cdot}46$ per cent respectively (Table 1). The deaths began three days after the inoculation of 1,000 $ID_{50}$ of virus and continued through to the 10th day (Fig. 1). The mean 50 per cent death time was $5{\cdot}1 \pm 0{\cdot}8$ days. The inoculation of 10 $ID_{50}$ increased the 50 per cent death time to $7{\cdot}2 \pm 1{\cdot}4$ days. The difference between the times was highly significant ($t = 4{\cdot}142$, $P < 0{\cdot}005 > 0{\cdot}001$).

TABLE 1

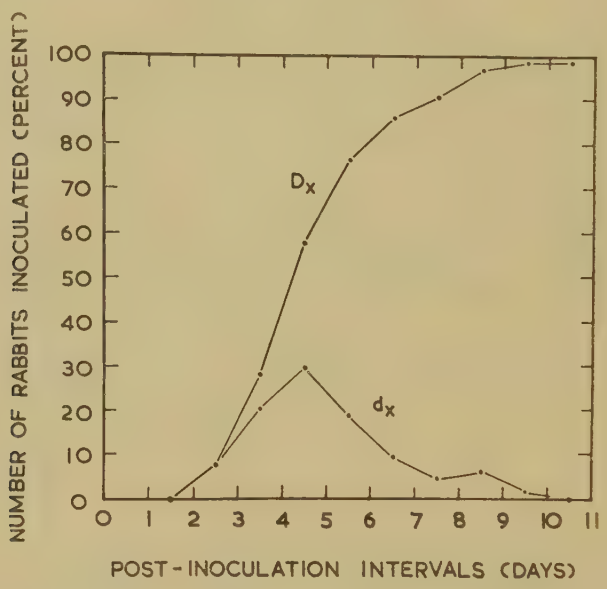
DEATH PATTERNS IN RABBIT POPULATIONS INOCULATED WITH LAPINISED RINDERPEST VIRUS

| *Days after inoculation* | *Dose of virus* | |
|---|---|---|
| | *10 $ID_{50}$* | *1,000 $ID_{50}$* |
| 0—1 | 0 | 0 |
| 1–2 | 0 | 0 |
| 2–3 | 0 | 8 |
| 3–4 | 4 | 22 |
| 4–5 | 5 | 32 |
| 5–6 | 13 | 20 |
| 6–7 | 13 | 10 |
| 7–8 | 12 | 5 |
| 8–9 | 6 | 7 |
| 9–10 | 4 | 2 |
| 10–11 | 0 | 0 |
| 11–12 | 2 | 0 |
| Number of rabbits inoculated | 64 | 108 |

Irrespective of the dose, the logarithm of the rate of mortality at day x and over was directly proportional to the post inoculation interval (Fig. 2). The regressions were highly significant ($P < 0{\cdot}001$), the variance ratios being 181·84 and 75·50 for Gompertz functions associated with the inoculation of 1,000 and 10 $ID_{50}$ of virus respectively. The slopes of the two functions were 0·0643 and 0·0604. The difference was not significant ($t = 0{\cdot}463$, $P > 0{\cdot}5$) and therefore the rates of mortality were similar. However, the means of the two

regressions differed and therefore the position of the lines differed. In other words deaths occurred sooner when the higher concentration of virus was inoculated.

Fig. 1

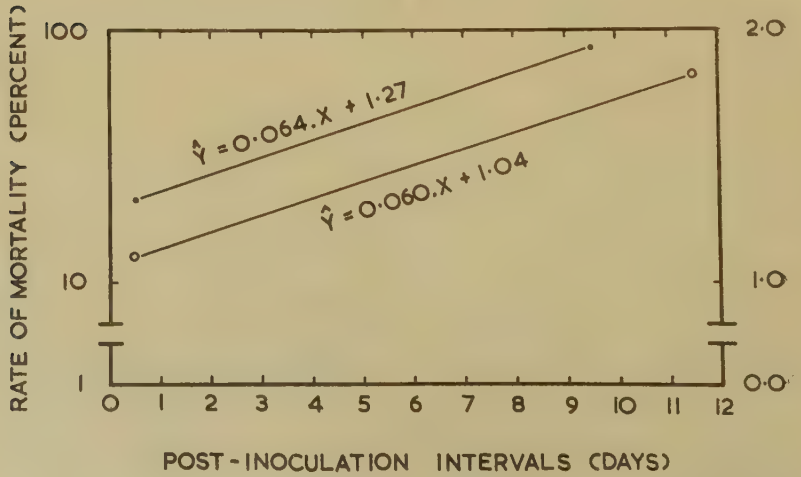



Percentage mortality in rabbits inoculated with lapinised rinderpest virus.

$d_x$ = deaths per day.

$$D_x = d_x + d_{x+1} + \ldots + d_\omega = \sum_x^W d_x$$

Fig. 2




Gompertz function for rabbits inoculated with 1000 $ID_{50}$ (●——●) and 10 $ID_{50}$ (○——○) lapinised rinderpest virus.

## DISCUSSION

Implicit in matters epidemiological is the assumption that the interaction between virus and host exhibits predictable characteristics. Some of these characteristics are influenced by the amount of virus entering the host, as shown, for example, by the inverse relationship between the dose and the length of the incubation period of rinderpest infection in rabbits (Scott and MacLeod, 1955). Others, such as the peak virus titre, are not related to the dose. We have here defined four more characteristics of lapinised rinderpest virus infection in the rabbit; the case mortality rate, the rate of mortality, the Gompertz function and the 50 per cent death time. The two former were not affected by the dose, but the two latter were inversely related to the size of the inoculum. The findings conform to the hypothesis that the response of the host requires a precise concentration of virus (Gard, 1943).

## CONCLUSIONS

The case mortality rate of rabbits inoculated with the Kabete substrain of the Nakamura III strain of lapinised rinderpest virus was $95{\cdot}96 \pm 2{\cdot}46$ per cent. The rate of mortality was not influenced by the amount of virus inoculated. The relationship between the logarithm of the rate of mortality and the post inoculation interval, i.e. the Gompertz function, was inversely proportional to the dose. Similarly, the dose influenced the 50 per cent death time.

## ACKNOWLEDGMENT

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[*Received for publication, July 11th,* 1958]

# STUDIES WITH RINDERPEST VIRUS IN TISSUE CULTURE

## I. Growth and Cytopathogenicity

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### INTRODUCTION

Rinderpest virus has been experimentally adapted to growth in goats and rabbits (see Brotherston, 1957 a, b), embryonated eggs (Shope, Griffith and Jenkins, 1946), and mice and hamsters (Scott, 1957). Of these hosts only the European rabbit reacts in a manner which can be recognized easily and specifically, unless the detection of complement-fixing antigens is accepted as a suitable indirect method of determining infection (Nakamura and Kishi, 1950, 1951; Boulanger, 1957). Rabbits have disadvantages in that they are expensive and difficult to produce and accommodate in the large numbers required, especially in the relatively under-developed, tropical countries where rinderpest control still remains a major problem for the veterinary services.

The present work was primarily undertaken to find an alternative, cheap system in which rinderpest virus would proliferate and produce easily recognizable cytopathogenic effects. With such a system the detection and titration of virus-neutralizing antibodies could be carried out on a much wider scale than previously practicable and investigations of the fundamental properties of the virus would be greatly facilitated. Studies reported here have shown that tissue culture methods not only provide a suitable answer to the initial problem; they have, in addition, thrown some light on the broad biological relationships of rinderpest to other viruses and, incidentally, furnished another attenuated strain suitable for the immunization of cattle. A preliminary account of some of our findings has already been published (Plowright and Ferris, 1957).

### MATERIALS AND METHODS

*Trypsin-dispersed cells.* Most of the work reported was carried out using monolayers of kidney cells, derived at first from 5 to 7 months bovine foetuses and later from calves slaughtered for veal at 1 to 6 weeks of age. Initially, trypsinised cell suspensions were produced by slight modifications of the technique of Younger (1954); it was subsequently found that very good results were regularly obtained by the less laborious procedure described by Bodian (1956) for monkey kidney and this has since been adopted as standard for both embryonic and post-natal kidneys.

After the usual washings, fragments of kidney cortex were agitated for 30 to 60 minutes at room temperature with 0·25 per cent trypsin

(Difco 1: 250) in Hank's saline using a magnetic stirrer geared down to about 200 r.p.m. When small fragments were observed to be detaching into the fluid the trypsin was discarded. Fresh trypsin was added and agitation was continued overnight at 4°C. No large fragments, apart from a few strands of connective tissue, remained by the following morning. The resulting cell clumps were filtered off through two layers of muslin, deposited by centrifugation, resuspended and washed twice in 5 per cent ox serum (O.S.) in Hank's saline. The packed cell volume was determined, and the cells were resuspended in growth medium at a concentration of 1/150 to 1/200 (v/v). All centrifugations were carried out for five minutes at 400 to 500 r.p.m. The procedure described has been employed for over 100 successive batches of calf kidneys, without a single failure; it was easily possible to obtain 10 ml. or more of packed cells from a single pair of kidneys, in one such trypsinization cycle.

Calf kidney cell suspensions (150 v/v in growth medium) stored for up to 3 or 4 days at 4°C, and used without further washing, produced complete cover of the glass within 4 to 5 days. There were no marked differences in the virus susceptibility of successive lots.

Trypsinised suspensions of cells from the kidneys of sheep and pig foetuses (near term) were produced by either of the two techniques described for bovine kidneys. Cultures were also prepared by Bodian's method from kidneys of lambs (1 to 3 months), goat kids (1 to 2 months), rabbits (6 weeks) and hamsters (6 weeks). In the case of rabbits and hamsters, disruption of the tissue was complete enough after 2½ to 3 hours treatment with trypsin at 4°C. Monolayers of cells from the testes of calves (1 to 8 months old) and lambs (1 to 3 months old) were prepared by a method already described (Ferris and Plowright, 1958). Plasma-clot tube cultures of fragments of rabbit embryonic skin, muscle and adult rabbit spleen were prepared by standard methods (Robbins, Weller and Enders, 1952).

*Culture vessels.* For virus passage and observations on its cytopathogenicity 8 cm. Carrel flasks were employed. Occasionally 4 oz. flat medicine bottles (U.G.B.) with metal screw-caps and rubber liners were used but their optical properties were poor. Monolayers on coverslips were grown and infected in 10 cm. pressed Petri dishes incubated in a chamber periodically flushed with 5 per cent carbon dioxide in air (Ferris and Plowright, 1958). In all these vessels 10 ml. of cell suspension was introduced and a similar volume of medium was replaced when fluids were changed. For growth curve experiments 75 ml. of cell suspension was seeded into Roux flasks; tube cultures for virus or antibody titrations each received 1 ml.

*Culture media.* The standard growth medium for all cell types had a basis of 0·5 per cent lactalbumin hydrolysate (Nutritional Biochemicals) in Hank's saline (Melnick and Riordan, 1952). After the early stages of this work 0·1 per cent bacteriological yeast extract (Difco) was also included as it appeared to prevent early degeneration and detachment of the cell sheet. At the first change of medium, usually after 2 to 4 days depending on the rate of growth and rapidity of fall of pH., an exactly similar basal medium was used with Earle's saline substituted for Hank's. This corresponds with the L.A.–Y.E. medium of Franklin, Rubin and Davis (1957). Subsequent complete changes of medium were carried out at intervals of 3 to 5 days. Pooled, unheated serum from adult

rinderpest-susceptible cattle (O.S.) was added in 5 per cent concentration to the growth medium for kidney cells from bovine and sheep foetuses, calves, goat kids, rabbits and hamsters. The same addition was made for calf testis cells, after a preliminary period when 10 per cent serum was found to produce a too prolific growth. For lamb and embryonic kidney cultures and also for lamb testis cells O.S. 10 per cent was used.

For virus production the maintenance medium routinely employed consisted of 5 per cent O.S. in L.A.–Y.E. Occasionally we tried L.A.–Y.E. without serum or with 10 per cent or 2 per cent O.S. and the results did not appear to differ materially. Unheated sera from some susceptible cattle appeared to depress the cytopathogenic effect of rinderpest virus and to result in lower titres. To overcome this difficulty such sera were later eliminated by virus titrations carried out concurrently in diluent free from serum and after mixing virus dilutions with equal volumes of the undiluted serum under test. The mixtures were left overnight at 4°C prior to inoculation into tubes. Only sera which did not depress the titre significantly were employed for media. Unheated, normal horse serum (H.S.) was not found to be satisfactory for the growth of bovine kidney or testis cells. It was sometimes used at 2, 5 or 10 per cent concentrations for virus production by established cultures and appeared to give comparable results to O.S. We have not investigated the matter in detail.

For some plasma-clot cultures and on some established monolayers, bovine allantoic and amniotic fluids (B.A.F.) with 5 to 10 per cent O.S. were tried (Enders, 1953). They were not so satisfactory for maintenance or virus production as the L.A.–Y.E. basal medium and in any case offered difficulties in their collection in large quantities.

All media used contain 100 units of sodium penicillin and 100$\gamma$ of streptomycin sulphate per ml.

*Virus strains.* All successful studies reported here were carried out with one virulent strain of bovine passaged virus, Kabete 'O', which has been maintained for over 40 years by subcutaneous inoculation of cattle with infected spleen suspensions (MacOwan, 1956). Its behaviour is very regular, cattle inoculated with large doses ($10^4$ $ID_{50}$ or more) showing high temperature reactions by the 2nd or 3rd day. It does not ordinarily produce any mouth lesions such as those characteristically associated with field strains of rinderpest, neither does it spread at all readily by contact to susceptible European-Zebu cattle (Brotherston, 1951 b; Plowright, 1952). The material used was infected bovine spleen, stored untreated at —20° to —25°C. and ground with sand to a 10 per cent suspension in phosphate-buffered saline, (P.B.S.) (Dulbecco and Vogt, 1954).

The goat-adapted virus was in its 614th goat passage, and consisted of a freeze-dried suspension of infected goat spleen. The lapinized virus preparations were spleen and lymph gland suspensions or freeze-dried, spleen, lymph gland and blood suspensions of rabbits infected with the Nakamura III strain of vaccine virus, which has undergone more than 700 rabbit passages. They were resuspended by grinding in a mortar with P.B.S. Infected tissue suspensions were centrifuged at 2000 r.p.m. for five minutes or more to free them from large tissue particles.

*Rinderpest immune sera.* Bovine immune serum obtained from cattle which had recovered from Kabete 'O' virus infection was stored at —25°C. and had an SN/50, in rabbits of about $10^{-2.5}$ (MacOwan, 1956).

Rabbit immune sera were obtained from two rabbits 22 days after inoculation of lapinized-avianized rinderpest virus (Nakamura and Miyamoto, 1953). Both showed typical mild thermal reactions. Immune goat sera were derived from two goats 11 days after inoculation of caprinized rinderpest virus. Immune serum was produced in hamsters by inoculation of a hamster-adapted strain of virus which produced no detectable symptoms in inoculated animals (Scott, 1957).

*Cattle and rabbit inoculations.* For the detection of bovine or caprinized strains of virus, grade cattle, 1½ to 3 years old, usually with a high proportion of European blood, were inoculated subcutaneously with 1·0 or 2 ml. doses of culture fluids. All survivor animals were tested for susceptibility by challenge of immunity with about $10^4$ $ID_{50}$ of 'O' strain virus. Lapinized virus preparations were injected into well-grown rabbits in doses of 1·0 ml. intravenously.

*Virus passage.* Two methods were used to infect cultures. The first (I) was that commonly employed, in which tissue suspensions or fluids from a previous passage were placed on established monolayers after discarding the medium. Adsorption took place for ½ to 2 hours at 36°C. and fresh maintenance medium was then added. The second method (II), which ultimately proved to be more satisfactory (v. infra), consisted of mixing virus-containing materials with trypsin-dispersed cells suspended in growth medium. At first the cell suspensions were concentrated ones 1/20, (v/v), which were diluted tenfold after allowing virus adsorption for a period of ½ to 1 hour at 36°C., with periodic or continuous agitation. This refinement was subsequently found to be unnecessary and the majority of later studies were carried out by mixing 1/150 (v/v) cell suspensions with one ninth their volume of infected fluid. They were then seeded immediately into the final containers and incubated at 36 ± 0·5°C. in a stationary position. Subcultures were normally carried out with undiluted fluids, harvested at 5 to 7 days after infection, i.e. when well-defined cytopathogenic effects were seen. Infected culture fluids were centrifuged at 2000 r.p.m. for five minutes to free them from cells or cellular debris in suspension. All data therefore refer solely to virus free in the fluid.

*Virus titrations.* Although virus preparations could be roughly titrated by determining the 50 per cent cytopathogenic end-point in established monolayers, it was difficult to detect typical focal "lesions" at higher dilutions, especially when cultures more than 5 to 6 days old were employed. All growth curve and quantitative neutralization studies were therefore carried out by mixing 0·1 ml. quantities of virus or virus-serum mixtures with 1·0 ml. of calf kidney-cell suspensions in the bottom of Pyrex culture tubes (16 × 150 mms.). For diluent L.A.–Y.E. medium without serum was used. At least five tubes were used per dilution and the cell-virus mixtures were well shaken. The tubes were closed by white rubber bungs (Esco) and incubated for four days sloped in a stationary position. At this time the medium was changed to L.A.–Y.E. with 5 per cent O.S. and the tubes were transferred to a roller drum. Low power microscopic examination was carried out at intervals between the 4th and 8th days and any tubes showing one or more distinct foci of cytopathic change were discarded as infected. Titres were calculated using the method of Thompson (1947), and are expressed as the reciprocal of the log of the 50 per cent end-point dilution.

*Stained preparations.* Coverslip preparations (7/8″ circles) of control and infected cultures were produced in 10 cm. Petri dishes (Ferris and Plowright, 1958). One or more were removed at intervals, rinsed in saline, fixed in Bouin's fluid for 10 to 20 minutes, washed with 70 per cent alcohol until free of picric acid and stained by Ehrlich's haematoxylin and eosin. It was found that changes in infected Petri dishes ran closely parallel to those in Carrel-flask or Roux-flask cultures of the same cell batch, inoculated at the same time with comparable doses of virus.

## RESULTS

### *Passage of 'O' Strain Virus in Bovine Kidney Cells*

After inoculation of established kidney-cell monolayers (Method I) with spleen suspensions containing about $10^4$ cattle $ID_{50}$ per ml. of the 'O' strain of virus, fluids harvested 3 and 7 days later still produced typical reactions in cattle. Three further passages were carried out with 3-day or 7-day fluid harvests. At the 4th passage, 3, 7 and 10-day fluids were infective to cattle at dilutions of $10^{-1}$, $10^4$ and $10^{-2}$ respectively. Further sub-inoculations were therefore carried out with 6 or 7-day fluids from the previous passage. As no cytopathogenic effect had been noted in unstained cultures up to this time, it was decided to infect cells as a suspension (Method II) and then note if virus proliferation interfered with their formation of a monolayer. The 5th to the 12th passages were carried out in this manner and distinct cytopathogenic effects were observed. At first these were detected from the 5th to 6th days onwards, but by the seventh passage they were evident at three to four days and no normal cells remained by about the 12th day after seeding.

The 13th to the 23rd passages were carried out using Method I to infect cultures. This at first appeared to be as satisfactory as a collateral line for which Method II was employed. Cytopathogenic effects were regularly observed as early as 18 to 48 hours after infection and complete destruction of the monolayer occurred in 10 to 14 days. By the 20th passage, however, the cytopathic changes were becoming delayed and did not proceed to completion in spite of the continual use of good 5 to 6 day cultures which had only just covered the glass and the attempted use of maintenance media (L.A.–Y.E.) containing 2 per cent or 10 per cent O.S., 2, 5 or 10 per cent H.S. and bovine amniotic or allantoic fluids with 5 per cent O.S. For the 24th passage Method II was again used. There was a rapid enhancement of the cytopathogenic effects observed and the method has since been used exclusively. To date the virus has reached its 50th passage in bovine kidney-cells; with undiluted viral inocula, effects clearly due to virus were visible on the 3rd day after seeding and very few normal cells were present by the 6th to 7th days.

Because of the obvious possibility of contamination by other cytopathogenic agents present in the serum or kidney cell suspensions, each virus passage was controlled by setting up cultures from

all the cell batches used in at least one 10 cm. Carrel-flask and one 10 cm. Petri-dish with coverslips. In the controls the virus-containing fluid was substituted by normal L.A.–Y.E. medium. The cultures were latterly retained for at least three weeks and frequent microscopic examinations were made. In 100 lots of uninoculated kidney cells, each derived usually from single calves, only one cytopathogenic agent was recovered which was apparently an "orphan" virus similar to others isolated from cattle tissues or secretions in the Union of South Africa and Southern Rhodesia (Alexander, Plowright and Haig, 1957).

*Early proof of the identity of the cytopathogenic agent.* Virus of the 6th kidney passage was diluted $10^{-1}$ or $10^{-2}$ and mixed with equal volumes of two pooled sera from four susceptible and two rinderpest-immune cattle respectively. After one hour incubation at 35°C. the mixtures were inoculated in 1 ml. quantities into trypsinised cell suspensions which were seeded into 8 cm. Carrel-flasks. Cytopathogenic effects occurred only in those flasks inoculated with susceptible-serum mixtures or in control cultures inoculated with virus only. Fluids harvested at the 12th day from cultures inoculated with the two serum-virus mixtures were each inoculated into two susceptible cattle. Those receiving material from cultures with cytopathic changes, i.e. with non-immune serum mixtures, developed hyperacute rinderpest but the others failed to do so. Hence the cytopathogenic agent was rinderpest virus. It was similarly shown that the cytopathogenic effect could be neutralized by the sera of rinderpest-immune rabbits, goats and hamsters, but not by the normal sera of these species.

*Growth of 'O' Strain Virus in Bovine Kidney Cells*

All growth-curve studies were carried out using two to four Roux-flask cultures. Both systems of viral culture were employed, i.e. Method I on two occasions and Method II four times. The virus was used at its 26th to 28th kidney cell passages.

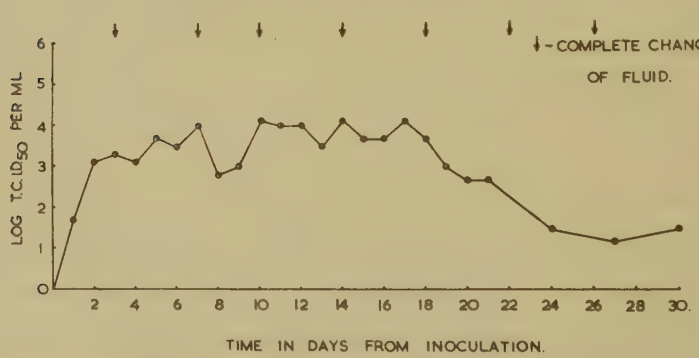
*Inoculation of established cultures (Method I).* Monolayers 6 to 7 days old were washed twice with 25 ml. P.B.S., and virus added. After two hours at 36°C. to allow adsorption, the viral inoculum was taken off and the monolayer washed three times with 25 ml. of prewarmed P.B.S. to remove unadsorbed virus. The inoculum was titrated before and at the end of the period of adsorption. 75 ml. of fresh, prewarmed medium was then added per flask. Aliquots of fluid (about 1 ml.) were withdrawn at daily and longer intervals, pooled, centrifuged at 2000 r.p.m. for five minutes and titrated in tube cultures. Complete changes of medium were made at 3 to 5-day intervals. Daily microscopic observations were made on the progression of cytopathic changes, particular attention being given to the proportion of the cell sheet involved and to the appearance and numbers of syncytia (v. infra). In addition, coverslips were removed at intervals from Petri-dishes infected in parallel with the

Roux-flasks. The results of one experiment are given in Text Fig. 1, these being comparable to those in the other experiment of this type.

Release of newly formed virus was demonstrable at 24 hours, peak titres of about $10^4$ per ml. being attained by the 7th day. The curves showed that release of virus to moderately high titre (3·5 or more) continued over a period of 18 days, there being a temporary fall at the 8th and 9th days, at which time detachment of single cells was probably at a maximum (v. infra). The first rapid rise of titre was associated with the presence of many rounded refractile cells and small syncytia with ill-defined edges. (Figs. 3 and 4). The second plateau of virus production was accompanied by the emergence and fusion of large syncytia which covered up to about 30 per cent of the available surface. The decline of virus titre which began on the 19th day was associated with evidence of degeneration and detachment of some of the multincleate formations (v. infra). Nevertheless, a steady titre of about 1·5 per ml. was maintained between the 24th and the 35th days, when the experiment was terminated.

TEXT FIGURE 1.

GROWTH OF RINDERPEST VIRUS IN ESTABLISHED MONOLAYERS OF BOVINE KIDNEY CELLS.



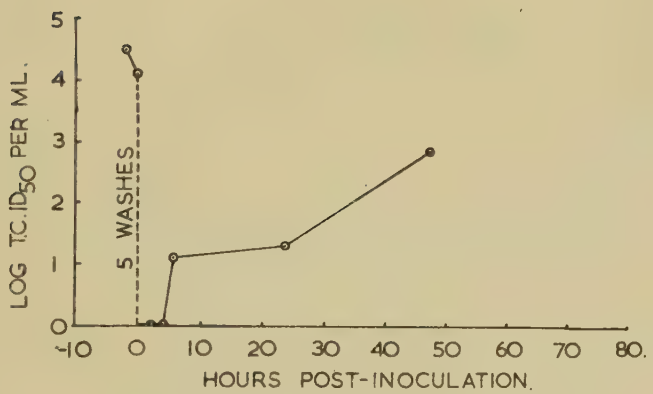

Virus adsorption and first release was also investigated in a similar experiment using three 6-day cultures in Roux-flasks. After a 2-hour adsorption period the monolayers were washed five times with 25 ml. P.B.S. and virus free in the fluid was sought at 2, 4, 6, 24 and 48 hours. First progeny or eluted virus was detected at six hours and increased to a titre of 2·9/ml. at 48 hours (Text Fig. 2). In the three experiments involving established monolayers the proportion of virus adsorbed during two hours at 37°C. from an inoculum of 7·0 to 10·0 ml. with an initial titre of from 3·9 to 4·7/ml., was 60 per cent to 84 per cent.

*Inoculation of trypsinised cell suspensions* (*Method II*). Four experiments were carried out using four separate batches of trypsin-dispersed cells. Virus with a titre of 3·7 to 4·5/ml. was mixed with

nine times its volume of cell suspension in final growth medium at 37°C. and dispensed into two to four Roux-flasks, together with Petri-dishes containing coverslips for microscopic study. In one instance (Text Fig. 4B), the virus-cell mixture was agitated for one hour at 37°C., on a magnetic stirrer, prior to dispensing, but this did not appear to accelerate virus adsorption. The viral inoculum and pooled aliquots of the infected cell suspensions or culture fluids were titrated at intervals. Prior to dilution they were centrifuged at 2000 r.p.m. for five minutes so that only free virus was measured. The usual microscopic observations were carried out.

TEXT FIGURE 2.

ADSORBTION AND RELEASE OF RINDERPEST VIRUS IN KIDNEY CELL MONOLAYERS.



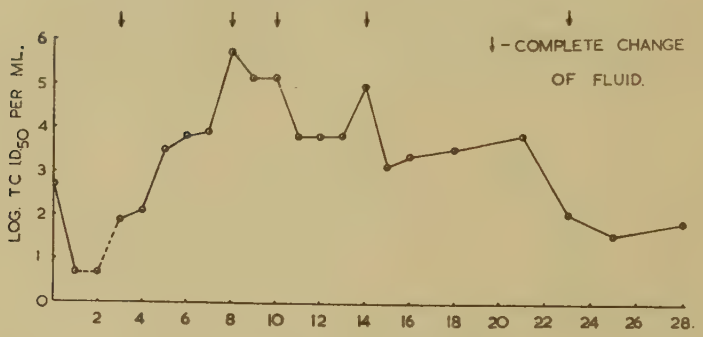

The adsorption of virus to cells, in four experiments, is shown in Text Figs. 4 and 5. In two instances (B and C), no fall in the titre of free virus was demonstrable during the first two hours. By the 5th to 8th hours 92 per cent to 97 per cent of the input virus was either adsorbed or inactivated in experiments A, B and C. Some free virus was always demonstrable at 24 hours, and in two of four cases (B and D) was also detected at 48 hours in undiluted fluid. More or less rapid rise of titre occurred on and after the third day and maximal titres of 4·5 to 6·0 were attained by the 5th to 8th days. Little fall occurred over the following 3 to 4 days. The results of one experiment (C) are shown in Text Fig. 3. A titre above $10^{-3}$ was maintained until the 21st day, after which it fell to a lower level, 1·5 or above, which remained steady until the end of the fourth week, when the experiment was terminated. The results of other growth-curve studies were similar, titres of free virus remaining between 1·5 to 2·5 up to 28 days after seeding.

In all these experiments involving infected cell suspensions, a

monolayer covered two-thirds or more of the available surface on the 3rd day; it was 80 per cent to 100 per cent complete by the fourth morning. Discrete foci of cytopathogenic change were usually detected on the 3rd day and these increased in number and coalesced so that about 60 per cent to 70 per cent or more of the cell sheet was involved by the 5th to 8th days. The rate of progression of these changes varied to some extent in a manner dependent on the size of the virus inoculum, but not obviously related to the maximal titres attained.

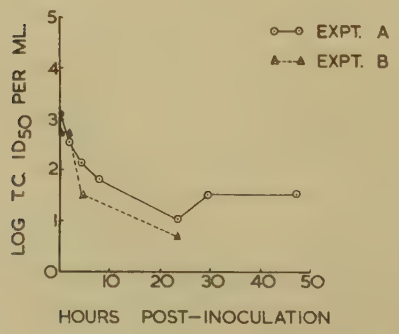
TEXT FIGURE 3.

GROWTH OF RINDERPEST VIRUS IN DEVELOPING MONOLAYER OF BOVINE KIDNEY CELLS.




TEXT FIGURE 4.

ADSORBTION OF RINDERPEST VIRUS TO DISPERSED BOVINE KIDNEY CELLS.



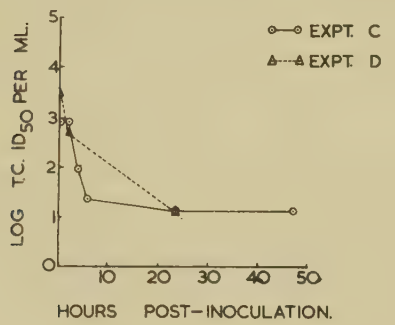

*Cytopathogenic Effect of Rinderpest Virus on Bovine Kidney Cells*

The effects of rinderpest virus on cultures were essentially the same whether inoculated before or after the formation of a monolayer; there were, however, differences in degree which appeared

to be related to the size of the inoculum and to the age and presumably the metabolic state of the cells. When newly-formed monolayers were infected the first changes were usually seen in situations where the periphery of adjacent cell islets had just fused, i.e. in "younger" cells which had most recently emerged from mitosis. When old-established cultures were used early changes were only detectable in the thinner parts, often at the periphery and such monolayers usually showed a chronic type of infection, observed by us up to eight weeks, in which destruction and regeneration remained focal and were roughly balanced. In the course of titrations, utilising cell suspensions, the only areas which needed to be examined in the higher dilution tubes were the edges of the growth, especially towards the bottom of the tube, since cytopathogenic changes occurred there with great regularity and seldom or never in the crowded central parts.

TEXT FIGURE 5

ADSORBTION OF RINDERPEST VIRUS TO DISPERSED BOVINE KIDNEY CELLS.




Infected cells reacted in two distinct ways; either they became rounded or stellate and showed increased refractility, or they formed multinucleate syncytia, the limits of which were difficult to see in either living or stained preparations. The first type of change usually predominated in the early stages of infection, especially when established monolayers were inoculated; the second was characteristic of the later stages of both types of infection, but predominated more rapidly in developing cultures. It is necessary, however, to point out that some of the well-demarcated, refractile and rounded elements, such as those in Figs. 2, 3, 4 and 7, contained two to four nuclei and were in reality small syncytia. They usually had one or more fine processes which were short and spiky or long, tenuous and sometimes beaded. These long processes often anastomosed with those from adjacent elements to form a loose meshwork (Fig. 3). Cells affected in this manner disintegrated into

shrunken fragments which detached from the glass within 2 to 3 days, being apparently killed by the virus. The time during which they were present in large and increasing numbers corresponded to the period of rapid rise of free virus titre, i.e. about one to five days with established, three to six days with developing monolayers. They presumably released virus at the time of their disruption. In fixed preparations the rounded, refractile cells retained basophilic dyes strongly and stained intensely. Because of the condensation of their cytoplasm it was difficult to see their nuclei clearly or any fine internal structure.

The syncytia which characterised cultures infected after establishment, were formed initially by the fusion of mononucleate cells. This is most clearly seen in Fig. 5, which shows how the individual cell membranes disappeared and the nuclei migrated into densely-packed clumps or rows. At the same time it became very difficult or impossible to see the boundaries of the cytoplasm which, for the most part, was extremely clear. Figs. 4 and 5 also show how this process of syncytium formation tended to spread from a series of "lacunae" into previously normal cell sheet, thus indicating an extension of the process by contiguity, rather than infection of many cells simultaneously via the fluid. Syncytia formed in this manner did not commonly show vacuolation, although it was observed occasionally. Small doses of virus inoculated into cell suspensions also produced this type of multinucleate formation in restricted cytopathic foci.

The syncytia formed at first in developing cultures infected with large viral inocula differed in that they were soon heavily vacuolated (Fig. 2). The vacuoles commenced as small rounded vesicles on the 4th and 5th days but rapidly enlarged and fused subsequently to displace the majority of the cytoplasm and compress the nuclei into a central, ill-defined clump. The "foamy" cells thus formed showed early signs of nuclear pyknosis and fragmentation and the majority detached within a further four to five days. Nevertheless, at about the time of maximal sustained virus titre, i.e. 6 to 10 days, they often covered more of the available area than any other cell type. Overlapping and following the degeneration and detachment of the "foamy" type syncytia, another cycle of syncytium-development began and extended up to about the 14th to 18th days (Figs. 9 to 12 inclusive). Certain cells with one, two or more nuclei appeared to survive the primary cycle of virus infection and destruction; these had no defined cell membrane but the cytoplasm included masses of a granular eosinophilic material outlined by a narrow, clear zone (Fig. 9). The syncytia gradually enlarged and fused to form bigger aggregates with increasing amounts of the cytoplasmic material. The nuclei commonly formed a central ring or clump and there was an impression that the cytoplasmic inclusions arose from the outer edge of the nuclear ring. Sometimes the nuclei were immediately surrounded by a zone of vacuoles (Fig. 11) which

separated them from the cytoplasmic inclusions. In other cases one syncytium contained a number of aggregations of nuclei or scattered single nuclei, each surrounded by a clear zone (Fig. 12). It was possible to find various stages in the fusion of two or more syncytia. Fig. 11 for example shows two large units with a scarcely discernible bridge connecting them. During the earlier stages of their formation, the cytoplasm of these multinucleate elements sometimes showed a peripheral fringe of coarse granular material, visible both in unstained and stained cultures; it helped in determining their limits.

After the 18th to 21st days an increasing proportion of the "secondary" syncytia showed pyknotic clumping of their nuclei and coarse cytoplasmic vacuolation which preceded their complete degeneration and detachment from the glass. Many, apparently fully-viable, still persisted into the fourth week and since no other cell type was present in significant numbers during the third and fourth weeks, it must be concluded that the long "tail" of virus production during this time was attributable to them. The cytoplasmic inclusion material, which was so prominent in these large syncytia, was also invariably formed, though in smaller quantities, in those developing in established kidney cultures (Figs. 4 to 6 inclusive) and in testis cultures from calves (Fig. 7). Its occurrence was found to be a reliable indicator of the presence of rinderpest virus and it was undoubtedly specific.

During the later stages, specific nuclear changes have also been constantly found in the multinucleate formations of rinderpest-infected cultures. These take the form of small, rounded or oval, eosinophilic inclusions surrounded by a partially clear halo (Fig. 8). The fact that the halo was not absolutely clear was probably due to the overlying and underlying normal parts of the nucleus. Single nuclei sometimes contained as many as four to five such inclusions, but there were usually one or two which were often very small and lay in different focal planes; it was therefore very easy to miss them. Fig. 13 shows an unusually large inclusion body in one nucleus and also the homogeneity in structure which usually characterised them. Fig. 14, on the contrary, illustrates the tendency to internal vacuolation which occurred in the larger bodies as they become older. The nucleoli were sometimes displaced peripherally by the inclusions (Figs. 8, 13 and 14) but remained essentially intact.

*Host-Cell Range of Culture-adapted Rinderpest Virus*

*Calf testis.* Fluid harvested at six days from the 6th bovine kidney passage was found to produce cytopathogenic effects in monolayers of calf testis cells. A series of 16 further passages was carried out in two to four day monolayers of the same type, fluids usually being harvested at the 5th to 7th days for subinoculation. At the 7th and 8th passage levels it was shown that the infection of trypsinised cell suspensions produced comparable results to the

inoculation of established monolayers. The former method offered little or no advantage, however, and was not adopted as routine.

There was a gradually increasing intensity and rapidity in the cytopathogenic effect of the virus, so that whilst some islets of normal cells still remained at the 11th to 14th days in the first six passages, destruction of the monolayer was thereafter virtually complete on the 4th to 7th days following inoculation. It was again noticeable that focal changes were first seen in the thinner areas of the cell sheet and were obscured where it was thicker and multilayered, even several days later. In unstained cultures there was a greater tendency than with kidney cells for the production of refractile, spindle-shaped or rounded masses, with long fine processes (Fig. 6). Larger syncytia often had a refractile, "glassy" appearance and their outlines were more clearly demarcated. In stained preparations the changes in testis cells were identical in all major respects with those described for kidney cells; there was usually, however, more tendency for infected fibroblasts to disintegrate directly into coarse, deeply-staining fragments. The cattle pathogenicity of virus passaged in bovine testis culture is mentioned in the second paper of this series (Plowright and Ferris, 1959).

*Sheep kidney.* Virus of the 24th bovine kidney passage was inoculated on to established monolayers of sheep embryonic kidney cells. Foci of typical cytopathogenic change were seen on the 3rd day and virtually all remaining cells were abnormal by the 7th day. In two further passages in the same type of culture the time to complete involvement was four to five days.

*Sheep testis.* Fluid from the 2nd sheep kidney passage was inoculated on to sheep testis cultures, which showed cytopathic changes extensive on the following day and practically complete on the 4th day. A further four passages were carried out by inoculating fully-grown monolayers with fluid harvested at the 4th to 7th days post-infection; the speed of their destruction by the virus was maintained. It is thus evident that kidney and testis cells from sheep show a susceptibility comparable to that of similar cells from cattle.

*Goat kidney.* The same inoculum used to infect embryonic sheep kidney cells was employed also for goat kidney cultures, which showed numerous focal changes from the 4th day onwards. On subinoculation of fluid to another series of cultures, only a restricted cytopathogenic effect was observed.

*Pig kidney.* Monolayers of pig embryonic kidney cells were inoculated with virus of the 26th calf kidney passage. Cytopathic changes were observed from the 2nd day following and were virtually complete on the 7th day. No subinoculation was carried out. Malmquist (personal communication) has subsequently confirmed the susceptibility of monolayers derived from adult pig kidney and an established line of similar cells.

In all the above-quoted cases fixed and stained coverslip preparations were available for microscopic observation. It was ascertained that multinucleate syncytia, with eosinophilic inclusion material in the cytoplasm and intranuclear inclusions of Cowdry type "B", were produced without exception.

*Hamster kidney.* The same inoculum as above produced ill-defined, focal "lesions" by the 3rd day. On the 10th day extensive and typical destruction had occurred. No microscopic preparations were available for infected hamster kidney.

*Rabbit kidney cells.* A single attempt to demonstrate the cytopathogenicity of culture-adapted virus for rabbit kidney monolayers failed. Inoculated cultures did not differ from controls at the 7th day.

*Failure of Goat-Adapted and Lapinized Virus to Grow in Monolayer Cultures*

In two experiments, 9 or 10-day monolayers of rabbit kidney cells were inoculated with about $10^{4.5}$ rabbit ID/50 of lapinized virus. In both no virus could be recovered from the fluid after 72 hours; in one instance cells disintegrated by two rapid cycles of freezing and thawing did not contain infective virus after 24 hours, and still later at 48 and 72 hours. Similarly, 5-day monolayers of bovine embryonic kidney failed to support the growth of a comparable inoculum, no virus being demonstrable in fluids harvested three days later. Plasma-clot cultures of fragments of adult rabbit spleen and embryonic skin-muscle tissue, all of which showed a good fibroblastic outgrowth, were also inoculated with lapinized virus but failed to show specific cytopathic changes in the following 13 to 18 days and fluids harvested at three and seven days were not infective to rabbits. Monolayers of rabbit testis cells did not support the growth of lapinized virus or show cytopathic changes detectable in stained coverslips in the 11 days following a virus inoculum of about $10^5$ rabbit ID/50. Fluids harvested at three and seven days were again non-infective for rabbits.

Goat kidney monolayers were inoculated at the 6th day with a goat spleen suspension containing about $10^4$ cattle ID/50 of caprinized rinderpest virus. Unadsorbed inoculum was removed after 18 hours and fluids were harvested and tested for cattle infectivity after four and eight days. No evidence of cytopathogenicity was seen and no virus was demonstrated in the medium. Monolayers of calf kidney cells likewise showed no evidence of being able to support the growth of this strain of virus, even after a single "blind" passage.

## DISCUSSION

The efforts described in this paper to adapt rinderpest virus to growth in tissue culture were not by any means the first. Daubney (1938) claimed a limited success with Rivers-type cultures of

embryonic and adult tissues of cattle, goats and rabbits. He also expressed the view that "a cheap and effective variant of reduced virulence for bovines will eventually be produced by this method". Takematsu and Morimoto (1954) reported the culture of lapinized virus in roller-tube cultures of rabbit lymph node, spleen and bone marrow, but no cytopathogenic effect was seen. Our failure to show persistence or growth of this strain in cultures of rabbit tissues is, therefore, somewhat surprising. Nakamura, Motohashi and Kishi, (1958) showed that the lapinized-avianized (L.A.) strain proliferated in suspended-fragment cultures of chick embryos, virus being detected and titrated by direct complement fixation tests or by subinoculation into eggs. Virus was then detected in the eggs by the C.F. reaction. Nakamura (1957) described the use of this culture system in detecting virus-neutralizing antibody, but the advantages of a culture system in which rinderpest virus can be titrated directly by its cytopathogenic end-point and without recourse to further serological tests, are obvious.

The cytopathogenic effect of rinderpest virus on cultured cells shows a close resemblance to that described for measles virus (Enders and Peebles, 1954; Cohen, Gordon, Rapp, Macauley and Buckley, 1955; Ruckle, 1957; Wright, 1957). Both produce multinucleate "giant cells" or syncytia in which there are intranuclear inclusions and large cytoplasmic accumulations of an eosinophilic material, surrounded by a clear halo and apparently specific (Milovanovic, Enders and Mitus, 1957). Rinderpest virus also causes some kidney cells and fibroblasts to become rounded, stellate or spindle-shaped and to acquire an increased refractility. Whilst this type of change is not recorded as occurring with measles virus in human or monkey kidney cells, it did become a prominent feature in human amnion cultures used for the serial transmission of the Edmonston strain (Milovanovic, *et al.*, 1957), and in chick embryo cultures infected with a chick-embryo adapted strain (Katz, Milovanovic and Enders, 1958).

It has been claimed (Reissig, Black and Melnik, 1956) that a predominantly spindle-cell change could be induced by measles virus in Hep-2 cells, if the latter were maintained after infection in Eagle's basal medium with the usual glutamine content; omission of the glutamine caused a return to the usual type of syncytium formation. Milavanovic, *et al.*, (1957) were inclined to discount this explanation in describing their results. We produced calf kidney monolayers by growth in Eagle's medium (Eagle, 1955) with and without glutamine, plus 5 per cent ox serum. These were infected with rinderpest virus and maintained in the same media; both series showed a similar cytopathogenic effect with very large numbers of syncytia and a few shrunken stellate cells (Figs. 5 and 6). The two types of cytopathic change discussed above occurred concurrently throughout this work with rinderpest and there has been no consistent tendency during 50 calf-kidney passages and

many in other cell types, for one to predominate at the expense of the other.

The cytoplasmic inclusions in measles syncytia probably include much viral antigen, since labelled antibody shows that specific fluorescence is usually limited to the cytoplasm in the earlier stages of infection (Cohen, *et al.*, 1955; Enders, 1956). We have not yet been able to carry out a similar investigation with rinderpest infected cultures. In measles Enders, Peebles, McCarthy, Milovanovic, Mitus and Holloway (1957) state that "Several days after the syncytia are apparent, eosinophilic inclusion bodies appear in most of the nuclei". The same delay in the appearance of intranuclear inclusions was noted with rinderpest, but with this virus the bodies tended to be very small and not delimited by a completely clear halo, or accompanied by such serious disruption of nuclear structure as a margination of chromatin. They could best be described as of type "B" described by Cowdry (1934) and were considered as primarily degenerative in origin. Cohen, *et al.*, (1955) did, however, find that bright fluorescing masses occurred in some nuclei in measles infected cultures, about two weeks after inoculation.

The host-cell range of cultured rinderpest virus recalls the extension in activity of measles virus to include cytopathogenicity for hamster, mouse and guinea-pig cells (Wright, 1957). In addition to ungulate cells—sheep, goat and pig—species which have long been known to be capable of supporting the growth of rinderpest virus in vivo, such as hamster cells, were also susceptible in vitro. Nevertheless, Scott (1957) has shown that the 'O' strain can be adapted to this species and to mice in vivo, but fails to produce any observable effect.

Infection of trypsinised cell suspensions, which we adopted as routine for the production and titration of cultured rinderpest virus, does not appear to have been employed for any other monolayer-virus system, although Lepine and Paccaud (1957) refer to it as offering no advantage with strains of "foamy" virus derived from monkey kidney. For rinderpest it had several merits in that (a) higher maximal titres of free virus were obtained: (b) a period of at least four to five days, required for the production of "clean" cultures which would have involved at least two extra fluid changes, was eliminated: (c) in virus or antibody titrations cytopathogenic changes were more easily detected at the end-point, by the examination of only a proportion of the available cell sheet—the expanding edge—and final readings could be made at the 8th day following seeding: this compares with about 14 days post-inoculation for measles (Peebles, McCarthy and Enders, 1957; Katz, *et al.*, 1958).

Our observations indicated that "older" cells were less likely to show the cytopathogenic effects of rinderpest and this is in accord with the experience of others with measles (Ruckle, 1957) and Theiler's "T.O." strain of mouse encephalomyelitis in mouse

tissues (von Falke, 1957). It does not, however, appear to be a commonly accepted explanation, although it is not infrequently noted that focal changes due to virus action tend to affect first the edges of the cell sheet, e.g. Enders and Peebles (1954) for measles, Lepine and Paccaud (1957) for "foamy" virus. There was certainly a difference in both the rapidity of extension and the morphological details of the effect, as between cells infected after they had formed a monolayer and those presumably infected during the time when they were undergoing frequent mitoses. Thus, by the 6th to 7th day highly vacuolated syncytia covered two-thirds or more of the available surface when trypsin-dispersed kidney cells were seeded together with large viral inocula into culture vessels. The numbers of syncytia and their degree of vacuolation were much reduced when the size of the inoculum was lowered or when established monolayers were infected. Since the peak titre of virus free in the fluid was also reduced in these circumstances it was tempting to associate these two phenomena and suggest that maximal virus yields come from the vacuolated syncytia. As Brown (1957) has pointed out with "normal" monkey-kidney viruses, vacuolation is not an essential concomitant of virus syncytium formation; in fact it disappeared at about the 4th passage in monkey kidney and the 3rd in human kidney cells. It was never prominent and faded out rapidly on passage in rabbit kidney monolayers.

Syncytium formation has now been recorded with a large number of viruses (for a review to 1954 see Enders, 1954; for mumps, Henle and Deinhardt, 1955; for monkey kidney agents, Rustigian, Johnson and Reihart, 1955; for CA viruses, Chanock, 1956; for N.D.V., Buthala and Mathews, 1957; and for agents associated with lumpy-skin disease of cattle, Alexander, *et al.*, 1957). We have also noted it in calf kidney and testis monolayers infected with vaccinia (unpublished observations). These viruses obviously belong to diverse groups. Franklin (1957) produced some evidence to show that syncytia formed in monolayers of chicken monocytes went on producing fowl plague virus for longer periods than mononuclear cells before they were destroyed. This also appeared to be the case with rinderpest virus, since the syncytia certainly produced virus for a period of several weeks without evidence of severe damage. In this context it is interesting to note that syncytium formation in culture does not of necessity imply virus action. Goldstein (1954) described syncytia from human monocytes growing on cellophane and has further examined these cultures cytochemically (Goldstein and McCormick, 1957). The results suggested that syncytium formation was not a degenerative process but was accompanied by signs of an increased metabolic activity, including nucleic acid synthesis. In view of these findings it is possible that the formation of multinucleate masses in virus infections may be a mechanism enabling the cells to support, without injury, a higher level of metabolic activity, including virus synthesis.

The finding of syncytia in rinderpest infected monolayers and the occurrence of amitotic "giant cells", not only in measles-infected cultures but also in the tissues of natural human cases (e.g. Warthin, 1931; Semsroth, 1939; Mulligan, 1944) led one of us (W.P.) to a histological examination of the organs of experimentally infected cattle. Multinucleate cells were observed to be numerous in the lymphoid and haemolymph tissues of cattle given hypervirulent culture virus; syncytia were also found in the lesions of the stratified squamous epithelia of the mouth cavity and, possibly, in the epithelia of the gastro-intestinal tract. This confirmed similar observations of Thiery (1956) but unlike this worker, we could not identify intracycloplasmic or intranuclear inclusions with certainty. Suggestive evidence of a close serological relationship between measles and rinderpest has recently been obtained (Plowright and Ferris—unpublished observations) by examining inactivated human sera for neutralizing activity against rinderpest virus in culture. Individual sera from 10 male adults were all found to contain antibody, detectable in the majority of cases up a final dilution of 1/64 to 1/256.

## CONCLUSIONS

A virulent bovine strain of rinderpest virus was adapted to growth in monolayers of bovine kidney cells and 50 passages were carried out in this cell type. Cytopathogenic effects became prominent in the 5th passage and were enhanced by infecting trypsinised cell suspensions at the time of seeding into the final vessels. When established monolayers were inoculated with large doses of culture virus, peak titres of about $10^4$ per ml. of fluid were attained by the 6th to 8th days; by infecting cell suspensions higher titres of $10^{4\cdot5}$ to $10^{6\cdot0}$ per ml. were reached by the 6th to 8th days after seeding. In each case a period of sustained virus production followed the original peak. This period lasted at least until the end of the 4th to 5th weeks, titres being $10^{3\cdot0}$ to $10^{3\cdot5}$ from about the 10th to 21st days, thereafter $10^{1\cdot0}$ to $10^{2\cdot5}$ per ml. of fluid.

Titrations of virus were carried out by mixing 0·1 ml. of falling dilutions with 1 ml. quantities of cell suspension in culture tubes. These were then allowed to form monolayers in the usual way. Final readings were made on the 8th day of incubation, small quantities of virus producing only focal lesions of microscopic dimensions, which were almost invariably towards the edge of the cell sheet. A similar technique was used in the detection and titration of neutralizing antibody.

Virus production was correlated with the cytopathogenic effects observed in living and stained preparations. Two main types of cell-change were seen: (a) the assumption of a round, elongated or stellate shape, together with increased refractility; (b) the formation of multinucleate "giant cells" or syncytia. The former predominated only at an early stage, syncytia presumably accounting for the long

"tail" of virus production. The syncytia showed increasing accumulations of a specific eosinophilic material in their cytoplasm, whilst small inclusions of type "B" (Cowdry, 1934) were frequent in their nuclei at a later stage.

Culture virus was also found to be cytopathogenic for cells derived from bovine and sheep testes and the kidneys of sheep, goats, pigs and hamsters. Two attenuated vaccine strains of rinderpest virus neither proliferated nor produced cytopathic effects in any cultures used.

The close similarity in the cultural behaviour of rinderpest and measles viruses is discussed. Both have certain common features in their natural pathology and many adult human sera were shown to neutralize rinderpest virus to moderately high titre.

ACKNOWLEDGMENTS

Thanks are due to Mr. W. G. MacLeod for producing the photomicrographs and the text figures, and to the Director, E.A.V.R.O., Mr. H. R. Binns, C.M.G., O.B.E., M.A., M.R.C.V.S. for help during the course of this work and for permission to publish.

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[*Received for publication, July 13th,* 1958]

## LEGENDS TO ILLUSTRATIONS

All preparations are of monolayers grown on coverslips, fixed in Bouin's fluid and stained by Ehrlich's haematoxylin and eosin.

Fig. 1. Normal 5-day monolayer of calf kidney cells (× 180).

Fig. 2. Infected 5-day culture of same batch, infected at time of seeding with large dose of culture-passage virus. Note especially the heavily vacuolated syncytia and darkly-staining, rounded cells (× 180).

Fig. 3. 11-day culture of calf kidney, infected at 4th day. Rounded, condensed cells with long fibrillary processes. Few normal cells top and bottom right (× 210).

Fig. 4. Same culture showing irregular syncytial masses which are not vacuolated (× 210).

Fig. 5. Monolayer (8-day) of calf kidney cells grown and maintained in Eagle's basal medium without glutamine. Two days post-infection, showing aggregation of nuclei to form syncytia with well-demarcated masses of eosinophilic material in cytoplasm (× 210).

Fig. 6. Higher magnification of inset in Fig. 5 (× 900).

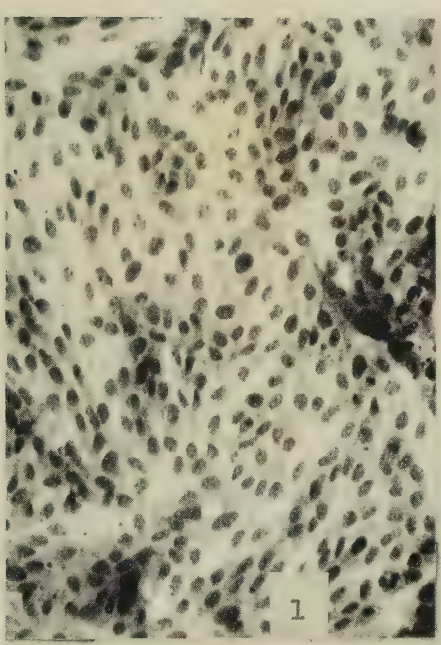
Fig. 7. 6-day culture of calf testis cells, 4 days post-infection. Note syncytia, rounded dark cells and cytoplasmic inclusions (× 210).

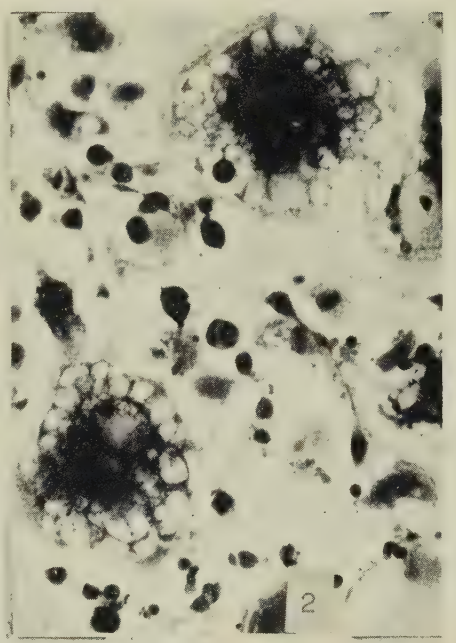
Fig. 8. Part of the nuclear aggregation in a large syncytium 14 days after seeding infected cells. To the right, dark masses of cytoplasmic inclusion material. Homogeneous intranuclear inclusions arrowed (× 900).

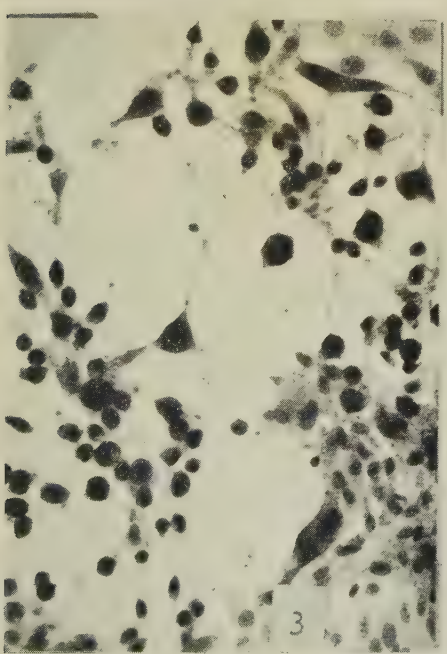
Figs. 9 to 12 inclusive. The development of very large syncytia in infected cultures of the same batch but incubated beyond the stage shown in Fig. 2, at 8, 12, 12, and 14 days respectively. The darkly staining areas in the cytoplasm, each with a distinct halo, represent specific inclusion material. Figs. 9 and 10 (× 210), Figs. 11 and 12 (× 180).

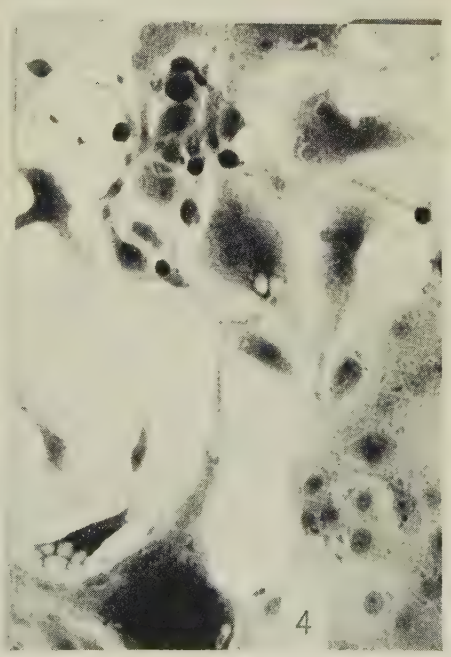
Fig. 13. Same culture as Fig. 9. A small syncytium with cytoplasmic and intranuclear inclusions (× 900).

Fig. 14. Same culture as Fig. 11. Nuclei of a syncytial formation, showing numerous inclusions with some internal vacuolation—arrowed (× 2000).

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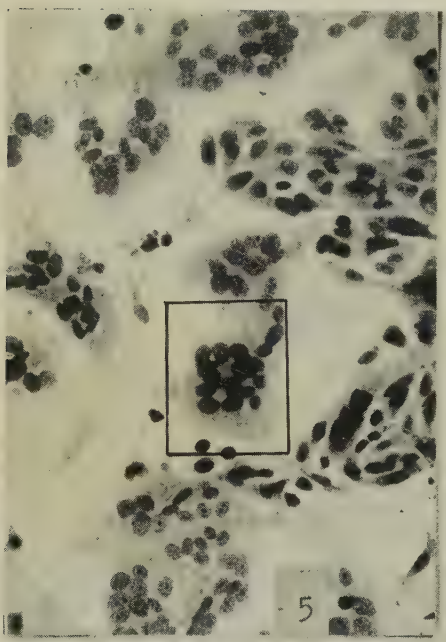
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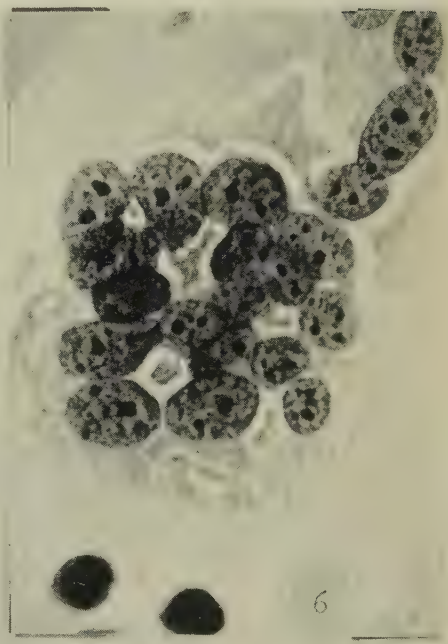
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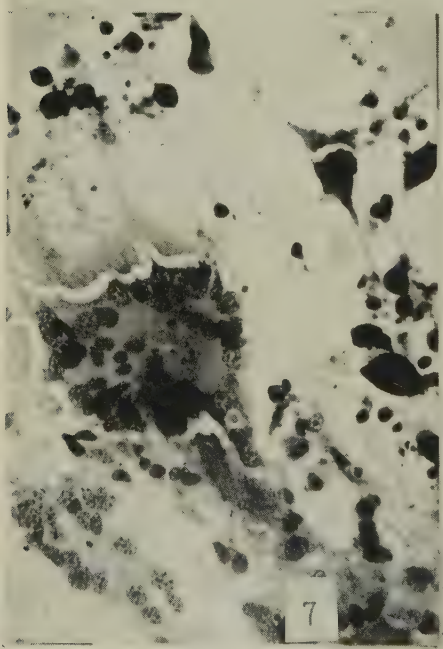
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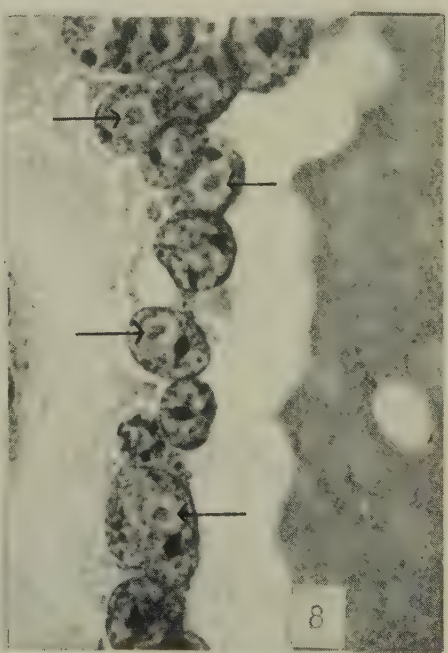
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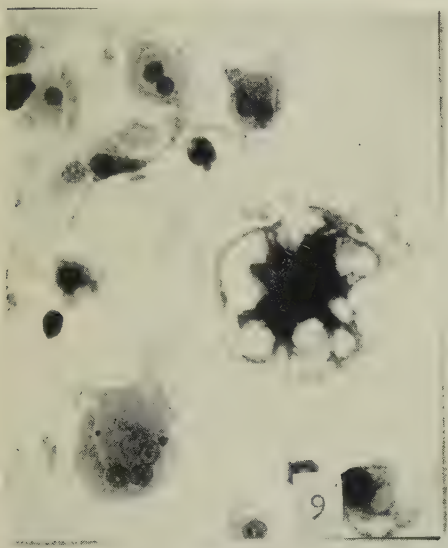
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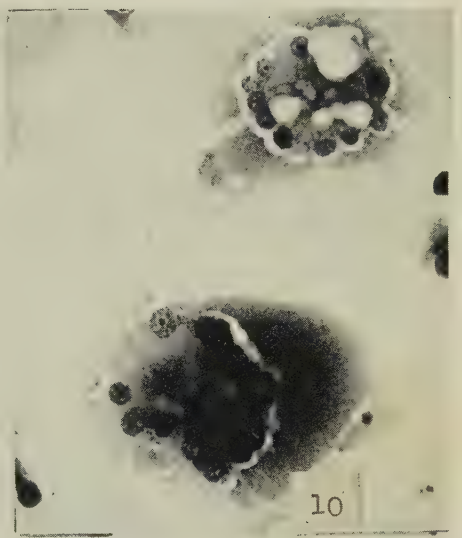
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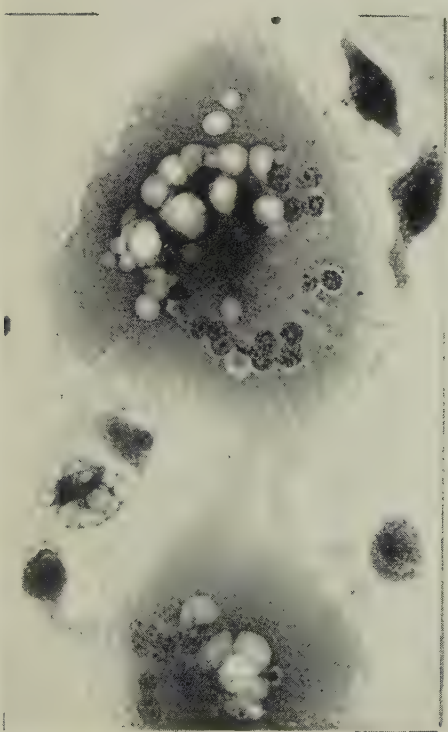

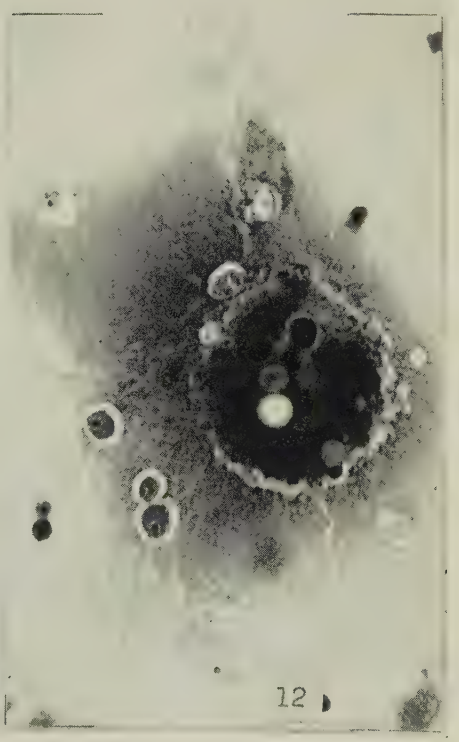
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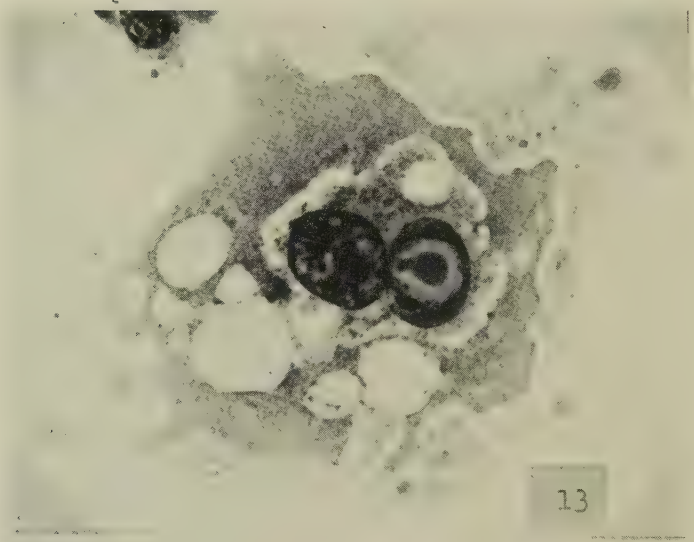
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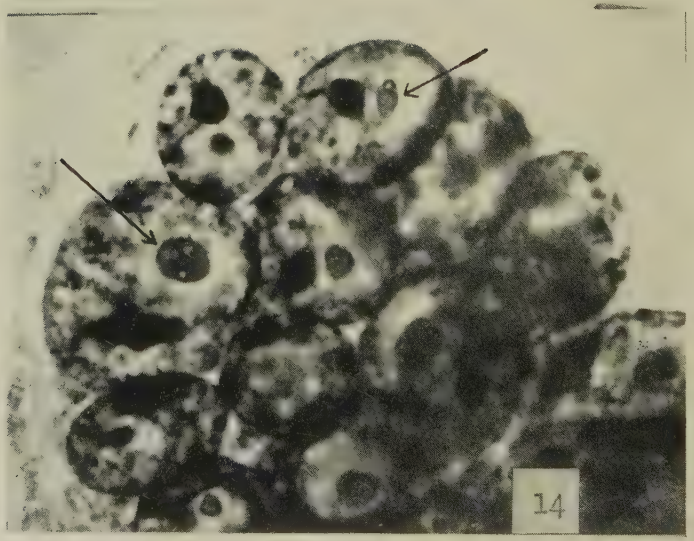
14

# STUDIES WITH RINDERPEST VIRUS IN TISSUE CULTURE

## II. Pathogenicity for Cattle of Culture-Passaged Virus

By

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### INTRODUCTION

A previous paper of this series described the adaptation of the Kabete 'O' strain of virulent rinderpest virus to growth in cultures of various ungulate and other cells. This communication describes the changes in its pathogenicity for cattle which occurred during passage in bovine kidney monolayers and mentions less extensive observations on virus passaged in calf testis cultures.

### MATERIALS AND METHODS

*Cultures.* The methods of preparation, maintenance and infection of bovine kidney monolayers have been already described. (Plowright and Ferris, 1959).

*Virus.* This was also described in the first paper of this series.

*Passage history.* To recapitulate briefly the history of the culture line in bovine kidney cells, passages 1 to 4 and 13 to 23 inclusive were carried out by the inoculation of established monolayers (Method I). Passages 5 to 12 and 24 to 45 inclusive were effected by the inoculation of trypsinised cell suspensions (Method II). Cultures for the first eight passages were derived from embryonic tissue, the rest from the kidneys of calves estimated to be about 2 to 10 weeks old. The inoculum always consisted of undiluted fluid from the previous passage, 1 ml. quantities being used for Carrel flasks or 4 oz. medicine flat bottles, and 7·5 ml. for Roux flasks. The medium used throughout for establishment of cultures consisted of Hank's saline with 0·5 per cent lactalbumin hydrolysate and, for the majority of the time, 0·1 per cent yeast extract. Five per cent pooled, unheated serum from adult cattle, subsequently shown to be susceptible to rinderpest, was always added. The medium used for changes had a basis of Earle's saline instead of Hank's; it also incorporated 5 per cent normal ox serum except in passages 21 and 22 when 5 per cent normal horse serum was used. The first medium change was carried out after 2 to 3 days and the second at 5 to 7 days. Fluid harvested at this latter time was used for subculture and the inoculation of cattle.

The testis-line of virus was initiated with fluid from the 6th kidney passage. Monolayers were inoculated at 2 to 4 days with 5 to 7 day fluids harvested from the previous passage. The media used were identical to the above.

*Virus and antibody titrations in culture.* The methods used for culture titrations of virus and antibody have also been described (Plowright and Ferris, 1959). Where comparisons of cattle and culture titres were made, 10 tubes of bovine kidney cells were used per dilution of virus, the

inoculum being 0·1 ml. Both series of inoculations were carried out within 1 hour, the dilutions being kept chilled.

*Experimental animals.* The cattle employed were usually grade steers with a more or less close resemblance to such European breeds as the Friesian, Guernsey or Ayrshire. The majority were between the ages of 18 and 36 months. All cattle used in titrations were "screened" prior to inoculation, to ascertain that they had no demonstrable neutralising antibody. For this purpose equal quantities of unheated serum and a virus sample diluted to contain an estimated $10^{1.5}$ to $10^{2.5}$ T.C. $ID_{50}$ per 0·05 ml., were mixed and left at 4°C. overnight. The following morning 0·1 ml. quantities of the mixtures were inoculated into 2 to 5 tubes containing 1·0 ml. of trypsinised calf kidney-cell suspension (1/150 v/v). The tubes were shaken and sloped in the usual manner. The complete protection of any tube at 8 days was regarded as evidence for the presence of antibody.

Cattle given culture virus were kept on daily (morning) temperature record and examined clinically at frequent intervals. The critical morning temperature was taken as 103°F. Particular attention was paid to the occurrence of anorexia, diarrhoea or dysentery, ocular and nasal discharges and mouth lesions. The dose of all virus preparations was 1 or 2 ml. subcutaneously behind the shoulder; titrations consisted of the inoculation of at least four tenfold dilutions, using 4 or 5 cattle per dilution.

The immunity of all survivor cattle was challenged by the subcutaneous inoculation, usually at 13 to 14 days after receiving attenuated virus, of 2 ml. of 10 per cent suspension of spleen from cattle killed at the height of the temperature reaction to the parent Kabete 'O' strain, estimated to contain at least $10^4$ cattle $ID._{50}$ The behaviour of susceptible cattle reacting to a similar dose of this virus was deduced from a study of animals used in these and other concurrent experiments.

## RESULTS

### *Initial Increase of Cattle Pathogenicity (Passages 1 to 10)*

Reference to Table 1, which summarises the results of cattle inoculations, shows that up to the 10th bovine kidney passage there was a definite increase in the pathogenicity of the 'O' strain of rinderpest virus. The minimum incubation period did not fall below 3 days, but this may have been due to relatively low-titre inocula; on the other hand there was a slight increase in the peak temperatures attained and rapid deaths cut short the period of pyrexia to a mean 3·62 days compared with 5·42 days for the parent strain. Mortality rose to practically 100 per cent from an initial level of about 60 per cent and the mean survival time fell to three days as compared with nine. The increase in mortality rate, at the 6th, 9th and 10th passage levels combined, was highly significant ($X^2 = 8{\cdot}551$, $p = {\cdot}00175$). Analysis of variance on the data for the survival times (9th and 10th passages pooled) showed that the reduction was significant at the 1 per cent level.

The lesions produced in infected cattle also showed considerable

TABLE 1

THE EFFECT OF PASSAGE OF KABETE 'O' RINDERPEST VIRUS IN BOVINE KIDNEY-CELL MONOLAYERS

| *No. of kidney passages* | *No. of cattle inoc'd.* | *Number showing pyrexia* | *Minimum incubation period (days)* | *Mean maximum temperature** | *Mean duration of pyrexia* (days)* | *Diarrhoea or dysentery* | *Mouth lesions* | *Mortality* | *Mean days to death†* |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 52 | 100% | 2 | 105·2°F. | 5·42 | 94·2% | 1/26 | 59·2% | 9·00 (29) |
| 1 | 4 | 4 | 3 | 105·6°F. | N.A. | + | Nil | N.A. | N.A. |
| 2 | 8 | 8 | 3 | 105·7°F. | N.A. | + | 5/8 | N.A. | N.A. |
| 4 | 8 | 8 | 3 | 105·5°F. | N.A. | + | 7/8 | N.A. | 3·0 (2) |
| 5 | 5 | 5 | 3 | 106·1°F. | N.A. | + | 5/5 | N.A. | N.A. |
| 6 | 10 | 10 | 3 | 105·6°F. | 3·86 (7) | + | 8/10 | 7/7 | 5·1 (7) |
| 9 | 10 | 10 | 3 | 105·5°F. | 3·62 (8) | + | 10/10 | 8/8 | 3·1 (8) |
| 10 | 3 | 3 | 3 | 105·4°F. | 4·0 | + | 2/3 | 3/3 | 3·3 (3) |
| 15 | 4 | 4 | 3 | 104·7°F. | 4·25 | + | 4/4 | 3/4 | 6·0 (3) |
| 16 | 5 | 5 | 3 | 105·1°F. | 5·0 | ? | ? | 3/5 | 8·6 (3) |
| 21 | 4 | 3 | 4 | 104·0°F. | 3·0 | Nil | Nil | Nil | Nil |
| 25 | 4 | 2 | 5 | 103·8°F. | 1·50 | ,, | ,, | ,, | — |
| 26 | 4 | Nil | — | — | — | ,, | ,, | ,, | — |
| 30 | 4 | 3 | 5 | 103·3°F. | 1·33 | ,, | ,, | ,, | — |

*=i.e. mean of all animals showing pyrexia. Critical morning temperature 103·0°F. throughout.
N.A.=information not available, usually due to slaughter of animals prior to end of the disease.
†=days to death calculated from first day of pyrexia. All figures in brackets refer to the number of animals involved.

modifications. Even at the 2nd passage, over 50 per cent of the animals had mouth lesions at death or when slaughtered on the 5th to 6th days post-inoculation. A closer examination in subsequent passages revealed that about 90 per cent developed typical foci of necrosis affecting several or all of the following epithelia, i.e. lips, gums, tongue, cheek papillae, hard and soft palates, and pharynx. The original strain of virus was noted for its failure to produce mouth lesions in any but a very small proportion of infected cattle—less than 4 per cent in the series reported here.

The watery diarrhoea or dysentery of the disease caused by unmodified 'O' virus, usually began on the 5th to 7th days following challenge inoculation, i.e. 3 to 5 days following the initial rise of temperature. The hypervirulent strain produced by culture passage caused a very violent form of dysentery, often with massive haemorrhage into the intestinal tract, and beginning 2 to 3 days after pyrexia. The post-mortem findings in these cases could be correlated with the clinical signs. Epithelial necrosis and desquamation, together with petechial and larger haemorrhages, were often very extensive, from the duodenum through to the rectum. Particularly surprising was the tendency for diffuse or semi-diffuse involvement of the mucosa of the small intestine, an organ often spared in cases caused by the original strain. Peyer's patches were commonly converted into clearcut haemorrhagic, denuded areas or showed an overlying, pseudomembranous deposit.

Another post-mortem finding of note was the intense congestion or haemorrhage and oedema which affected the lymph nodes, especially those of the head and neck region (submaxillary, pharyngeal, parotid and prescapular). Some of these also showed areas of macroscopic necrosis, confirmed on histological study. Such lesions were found with a much reduced frequency in 'O' strain infections. MacLeod and Plowright (1958) compared the haematological reactions of cattle given 9th kidney passage virus and the unmodified 'O' strain. The former caused a more rapid initial leucopenia, affecting especially the lymphocytes and eosinophils; in addition there was a marked neutrophil leucocytosis on the last 2 to 3 days of the fatal disease.

Finally, culture-passaged virus showed a considerable increase in its invasiveness for cattle. Brotherston (1951b), Plowright (1952) and MacOwan (1956) have all noted the great irregularity of contact transmission of the original strain and MacOwan (1956) gives the incubation period for such infections as $11{\cdot}7 \pm 1{\cdot}8$ days. In one experiment involving the titration of 9th kidney passage virus and before this enhanced invasiveness was observed, 32 cattle were housed in a series of 6 adjacent and non-isolated loose-boxes. 10 animals were thought to have been infected as the result of inoculation but a further 20 (91 per cent of the remainder) acquired the disease by contact during the following 26 days. Assuming that the earliest excretion of virus by any inoculated animal was 24 hours

prior to pyrexia, the mean contact incubation period was 9·9 days, a figure which falls within the range given by MacOwan (1956). Individual periods varied from 5 to 15 days, except in one animal which only sickened after 26 days. The minimum contact incubation period was similarly calculated, in a subsequent experiment with 15th passage virus, to be 4 days.

In the large experiment mentioned above it was found that later contact cases survived longer, showed less severe symptoms and failed to infect further susceptible animals introduced into the infected environment.

*Progressive Decrease of Cattle Pathogenicity (Passages 10 to 45)*

Between the 10th and 16th kidney passages there was some decline in virulence of culture virus. This was shown by a reduction in mortality, and the recovery of occasional animals after a prolonged course of disease. The figures available for the 16th passage, in fact, approximate closely to those calculated for the unmodified 'O' strain. Between the 16th and 21st passages a very striking reduction in pathogenicity occurred, so that from the latter onwards no overt clinical signs attributable to the virus were ever observed. With large inocula, as represented by undiluted culture fluids, of the 21st to 30th passages, up to 75 per cent of inoculated animals still showed mild temperature reactions, but this was the only detectable change and somewhat irregular in occurrence. The minimum time to onset of pyrexia showed a further progressive increase to 7 or 8 days post-inoculation at the 39th to 45th passage level and the maximum duration was 3 days. Reference to Table 2 shows that immunising infections with small doses of virus caused less frequent temperature reactions than when larger inocula were employed. Of a total of 60 cattle given $10^{2\cdot5}$ or more cattle $ID_{50}$, 23·3 per cent showed pyrexia, whilst of 36 immunised by less than $10^{2\cdot5}$ $ID_{50}$ only 5·5 per cent reacted. ($X^2=3\cdot96$, $p=0\cdot023$).

Another important change in the character of culture-attenuated virus was its inability to spread by contact. Susceptible cattle housed in the same loose-boxes with those given 21st or later passage culture virus did not become febrile or acquire "silent" immunising infections in periods of 13 to 16 days.

*Correlation of Cattle and Culture Titres of Attenuated Virus*

It was to be expected that increasing adaptation to cultural growth and reduced cattle pathogenicity would be associated with an increasing disproportion between the sensitivity of kidney cultures and that of cattle in detecting virus. Table 3 shows that this was not the case and provides evidence for the consistent accuracy of culture titrations, carried out by the inoculation of trypsinised cell suspensions. It should be stressed that the cattle titres were all obtained by inoculating groups of 4 to 5 cattle per dilution and subsequently challenging their immunity with un-

TABLE 2

TEMPERATURE REACTIONS OF CATTLE IMMUNISED BY 34TH TO 45TH PASSAGE VIRUS

| *Passage level* | *Log dose of virus (cattle ID/50)* | | | | | *Minimum incubation period* | *Mean maximum temperature* | *Mean duration of pyrexia (days)* |
|---|---|---|---|---|---|---|---|---|
| | 5·5 | 4·5 | 3·5 | 2·5 | <2·5 | | | |
| 34 | — | 2/4 | 2/4 | 0/4 | 0/7 | 5 | 103·8°F. | 2·0 |
| 36 | 1/4 | 0/4 | 3/4 | 2/4 | 1/4 | 5 | 103·8°F. | 2·0 |
| 39 | — | — | — | 2/4 | 0/9 | 7 | 103·0°F. | 1·5 |
| 40 | — | — | 0/24 | — | 1/7 | 7 | 103·0°F. | 1·0 |
| 45 | — | — | — | 2/4 | 0/9 | 8 | 103·4°F. | 2·0 |
| Totals | 1/4 | 2/8 | 5/32 | 6/16 | 2/36 | — | — | — |
| % Reactors | 25 | 25 | 15·6 | 37·5 | 5·5 | — | — | — |

*Critical morning temperature throughout 103·0°F.

modified 'O' strain virus. All animals which were immunised then showed no reaction whatsoever, those not protected developed typical severe rinderpest, commencing on the 2nd or 3rd day following challenge. Unfortunately no figures are available for simultaneous titration of low-passage virus, but it was demonstrated that peak infectivity of fluids, harvested at the 6th to 7th days after infection during the 4th, 5th and 6th passages, was of the order of $10^4$ cattle $ID_{50}$ per ml. This was before the virus could be regarded, on a basis of its cytopathogenicity, as fully adapted to cultural growth.

TABLE 3

CORRELATION OF CATTLE AND CULTURE TITRES OF KIDNEY-PASSAGED VIRUS

| *Passage level* | *Culture titre* (per ml. fluid)* | *Cattle titre* (per ml. fluid)* |
|---|---|---|
| BK/34 | 5·1 | >5·2 |
| BK/36 | 6·0 | >6·2 |
| BK/39 | 5·2 | 5·7 |
| BK/40 | 5·8 | 5·7 |
| BK/45 | 5·6 | 5·5 |

*Expressed as reciprocal of log of 50% end-point, calculated by the method of Thompson (1947).

*Interference by the Culture-Attenuated Strain with Subsequent Challenge by Virulent Virus*

Two experiments were conducted to find the time after inoculation at which cattle were protected by culture virus of the 39th or 46th generations. The results are given together in Table 4. It is apparent that even large doses of the attenuated strain did not protect against severe challenge until the 3rd day following inoculation. In the second experiment, where each animal received $10^{4.5}$ $ID_{50}$ of culture virus, sera were collected immediately prior to challenge and later examined for the presence of neutralising antibody. Up to and including the 5th day none could be detected in undiluted sera and the protection afforded was therefore regarded as due to specific exclusion or interference. At the 6th day neutralising activity was detected in the sera of both animals. The immunity of all non-reactor cattle in this experiment was again tested 14 days after the initial inoculations. All were shown to be solidly resistant to challenge.

*Serological Response of Cattle to Attenuated Culture Virus*

Serum was obtained from cattle used in virus titrations, at 13 to 14 days after the initial inoculations and immediately prior to immunity challenge. The samples were tested for neutralising

antibody at two levels only, i.e. at 1/2 and 1/10 final dilution, against $10^{1.5}$ to $10^{2.5}$ T.C. $ID_{50}$ of virus, using five tubes per dilution. The results for cattle resistant to challenge are given in Table 5. It is evident that smaller doses of attenuated virus either did not produce such a high level of neutralising antibody or that peak antibody titres had not been reached on the 13th to 14th days, when the serum samples were obtained. It was surprising to find that over 40 per cent of the undiluted sera of animals which received minimal doses of culture virus did not at this time completely neutralise in vitro $10^{1.5}$ to $10^{2.5}$ T.C. $ID_{50}$ of the same strain. But not one of the cattle showed any reaction whatsoever to challenge.

TABLE 4

INTERFERENCE BY ATTENUATED VIRUS WITH CHALLENGE BY VIRULENT 'O' STRAIN RINDERPEST

| *Days to challenge** | *Dose of culture virus (log T.C. ID/50)* | *No. of cattle* | *Antibody at challenge* | | *Reaction to challenge* | |
|---|---|---|---|---|---|---|
| 1 | 2·9 | 2 | N.T. | N.T. | + | + |
| 2 | 2·9 | 2 | N.T. | N.T. | + | + |
| 3 | 2·9 | 2 | N.T. | N.T. | + | + |
| Ditto. | 4·5 | 2 | — | — | + | — |
| 4 | 2·9 | 2 | N.T. | N.T. | + | + |
| Ditto. | 4·5 | 2 | — | — | — | — |
| 5 | 2·5 | 1 | N.T. | | + | |
| Ditto. | 2·9 | 1 | N.T. | | — | |
| Ditto. | 4·5 | 2 | — | — | — | — |
| 6 | 4·5 | 2 | + | + | — | — |

*c. $10^5$ cattle ID/50's subcutaneously.
N.T.=not tested.

TABLE 5

ANTIGENIC RESPONSE OF CATTLE IMMUNISED BY CULTURE ATTENUATED VIRUS*

| *Virus dose (T.C. ID/50)* | *No. of cattle* | *Complete neutralisation* | | *% positive at 1/2* | *% positive at 1/10* |
|---|---|---|---|---|---|
| | | *at 1/2* | *at 1/10* | | |
| $<10$ | 12 | 7 | 7 | 58 | 58 |
| $>10$ | 16 | 13 | 9 | 81 | 56 |
| $>10^2$ | 12 | 12 | 8 | 100 | 66 |
| $>10^3$ | 8 | 8 | 7 | Ditto | 87 |
| $>10^4$ | 4 | 4 | 4 | Ditto | 100 |
| $>10^5$ | 4 | 4 | 4 | Ditto | Ditto |

*The virus had undergone 36 to 45 calf kidney passages.

TABLE 6

EFFECT OF PASSAGE OF CULTURE VIRUS IN BOVINE TESTIS MONOLAYERS

| *Number of passages* | *Number of cattle inoculated* | *Number showing pyrexia* | *Minimal incubation period* | *Mean maximum temperature* | *Mean duration pyrexia (days)* | *Diarrhoea or dysentery* | *Mouth lesions* | *Mortality* | *Mean days to death* |
|---|---|---|---|---|---|---|---|---|---|
| 5 | 2 | 2 | 3 days | 105·1°F. | 4·0 | 2/2 | 2/2 | 2/2 | 3 |
| 10 | 4 | 4 | Ditto. | 106·2°F. | 5·2 | 2/4 | 2/4 | 2/4 | 5 |

*Effect of Passage of Rinderpest Virus in Calf Testis Monolayers*

Table 6 shows the results of inoculating cattle with fluids from the 5th and 10th passages in cultures of calf testis cells. Although the number of cattle available for test was small, there were definite indications of a decline in pathogenicity for cattle between these two levels. Two of four cattle given 10th passage virus recovered without showing diarrhoea, dysentery or mouth lesions. The duration of pyrexia in these two animals was 5 and 7 days respectively, these figures and the mortality roughly corresponding to those noted in Table 1 for the 16th direct kidney passage.

## DISCUSSION

It has been shown that passage of the 'O' strain of rinderpest virus in bovine kidney tissue cultures, resulted at first in an enhanced virulence for cattle, which was independent of the dose of virus inoculated. Increased pathogenicity was certainly observed between the 4th and 10th passage levels, the peak being probably at about the 9th and 10th passages. We are aware of very few similar observations on other viruses maintained in tissue culture. Topacio and Hyde (1932) reported an apparent exaltation in virulence for rabbits of Virus III, after growth in plasma clot cultures of rabbit testis. Later Ivanovics and Hyde (1936) implied that this might have been due to the fact that culture titres of virus were considerably higher than those in tissue infected in vivo.

Mayr and Schwöbel (1957) and Mayr (1957) described the changes which occurred in strains of Teschen disease virus on passage in pig kidney cultures; one strain (Tirol), which had been passaged 10 times by intracerebral inoculation of pigs, did not have much pathogenicity when injected by other parenteral routes. After 6 culture passages, however, it not only produced paralysis in a considerable proportion of pigs inoculated intramuscularly but also in those given large doses of virus by the mouth. A similar state of affairs was revealed with the rinderpest strain used in this work, since culture passage at first favoured the multiplication of virus particles with a natural invasiveness resembling that of field strains, whereas infectivity by natural routes had been largely lost in the parent strain. The ability to spread by contact may be associated with the production of patent lesions of the mouth and pharyngeal region. At any rate these became very frequent with culture passage and were seldom or never seen with unmodified 'O' virus. On the other hand, both strains produced frequent necrosis and erosion of the mucosa of the nasal cavities, lesions which would be expected to favour respiratory excretion of virus, as droplets capable of being inhaled by in-contact cattle.

The loss of virulence which occurred on culture passage was extremely rapid. At the 16th passage the virus produced a mortality comparable to that of the parent strain (60 per cent). At the 21st passage and thereafter no overt clinical signs were ever seen in

inoculated cattle but they still showed a high proportion of very mild febrile reactions if given large doses of virus. It is perhaps significant that the attenuation occurred predominantly during the time when established monolayers were used (passages 13 to 23 inclusive), and not cell suspensions. Against this is the fact that some exaltation of virulence occurred during the first four passages, which were also carried out by the same method. The rapidity of attenuation in tissue culture contrasts strongly with the prolonged passage of this same strain often necessary to achieve the same or less result in other unusual hosts. For example, after 250 to 400 subinoculations in goats it still caused marked temperature reactions, occasional symptoms and up to 2 per cent deaths in East African zebu cattle, which were regarded as naturally more resistant than the grade animals used here (Daubney, 1949). After 26 subcultures on the C.A.M. of chick embryos and 55 by the yolk sac route, it still produced clinical reactions in calves (Jenkins and Shope, 1946). McKercher (1957) found that loss of virulence of the 'O' strain virus commenced at about the 30th and was "complete" after about 40 C.A.M. passages. The loss of invasiveness, which accompanied falling pathogenicity for cattle, is in accord with previous observations on attenuated strains used for immunisation [for references to goat-adapted and lapinised virus see Brotherston, 1957a and b, for avianised virus, Jenkins and Shope (1946) and Hale and Walker (1946)].

The data obtained on interference by culture-attenuated virus with subsequent infection by the virulent parent strain, showed as expected that the minimum time for the establishment of protection varied according to the dose of the excluding virus from 3 to 5 or more days. It is probable that the interval depends on the time to appearance of viraemia and generalised infection. For example, with the goat-adapted strain of virus it is 48 hours (Pfaff, 1938), with lapinised virus it is between 84 and 108 hours (Brotherston, 1951a), these figures corresponding with an "eclipse" phase in cattle of 2 days and 4 days respectively (MacOwan, 1956).

Culture-attenuated virus offers certain advantages over many others so far as its use as a vaccine is concerned. It can be titrated very cheaply and reliably and it is regularly possible to produce fluids with an immunising titre in cattle above $10^5$/ml. An experiment to find the duration of immunity following inoculation of culture-passaged virus is in progress. Four cattle challenged 6 months after receiving $10^4$ cattle $ID_{50}$ of culture virus were all solidly immune. The decline of serum neutralising antibody is also being investigated.

## CONCLUSIONS

The changes in pathogenicity which occurred when a virulent bovine strain of rinderpest virus was passaged in bovine kidney monolayers are described.

In the first 10 culture generations there was an enhancement of virulence manifested by increased mortality rate, a decreased time to death and gastro-intestinal lesions of great severity. Mouth lesions, which were originally rarely seen, became extremely common and contact infection, although very irregularly produced by the parent strain, took place very readily.

Between the 16th and 21st passages a marked reduction in pathogenicity occurred. At the latter level and thereafter up to the 45th passage no overt clinical signs were ever detected, but cattle were immunised against subsequent challenge by virulent virus. A proportion of the cattle thus immunised showed mild temperature reactions, the frequency of which depended on the dose of the attenuated strain.

The immunising titres of culture-virus preparations were comparable to titres determined by the cytopathogenic end-point in tubes. Interference was demonstrated between the attenuated and virulent strains, the time to protection varying from 3 to 5 or more days according to the dose of the excluding agent. Neutralising antibodies were investigated in the sera of cattle given culture virus 13 to 14 days previously.

The conditions of culture in which attenuation occurred are detailed and discussed.

ACKNOWLEDGMENTS

We are indebted to Mr. G. J. Knight, F.I.M.L.T., for statistical analyses. Sincere thanks are due to the Director, E.A.V.R.O., Mr. H. R. Binns, C.M.G., O.B.E., M.A., M.R.C.V.S., for constant encouragement and help during the course of this work and for permission to publish.

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[*Received for publication, July 13th,* 1958]

# SOME OBSERVATIONS ON THE HISTOPATHOLOGY OF ALDRIN POISONING IN LAMBS

By

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## INTRODUCTION

Aldrin is a chlorinated hydrocarbon insecticide which has been used in forms suitable for application to livestock but is now more generally available as preparations intended to be used as pesticides for the protection of crops. If incorrectly used it may be poisonous to animals and man. Acute poisoning of farm animals by experimentally administered doses of the chlorinated hydrocarbons has been described by Radeleff, Woodard, Nickerson and Bushland (1955).

In a commercial flock some 40 per cent. of the lambs, aged about two months, were affected with contagious pustular dermatitis. The lesions were moderately severe and situated about the mouth and nose. The owner, without seeking advice, considered that aldrin was indicated in treatment and, having available a concentrated aldrin-containing preparation for crop protection, he applied this to the muzzles of all the lambs of the flock, affected and healthy alike. Of the 107 lambs treated all but two died. The occurrence has been reported by Pearson, Baird and Todd (1958) who described the field and clinical aspects together with the gross pathology. As lambs died at intervals of up to 15 days following the application of the aldrin an opportunity arose of studying the histopathological processes in a serial fashion.

## MATERIALS AND METHODS

Organs were selected from 7 lambs, 2 of which died 24 hours after application of the aldrin (lambs 1 and 2) and one each which died on the fifth, sixth, seventh, ninth and fifteenth days (lambs 3 to 7 respectively). The brain and pieces of liver, kidney, spleen and lung were fixed in 10 per cent formol-saline or in Bouin's fluid. Paraffin blocks were made and cut and, where necessary, frozen sections were also cut. The parts of the brain which were sectioned were the anterior dorsal part of the cerebrum, the anterior quadrate body, the vermis of the cerebellum and the medulla oblongata. The main staining method was haematoxylin and eosin. Subsidiary methods were van Gieson, Sudan IV, periodic acid-Schiff, Best's carmine and Spielmeyer's method for myelin.

## RESULTS

### *Brain*

*Lambs 1 and 2.* The lesions in both brains were of the same type and distribution, but in lamb 2 the changes were slightly less

severe. In the forebrain there was a moderately severe, diffuse gliosis especially in the white matter, while in the white matter there were a number of rather large foci of small round cells some of which exhibited fragmentation of the nucleus (Fig. 1). Elsewhere many of the oligodendroglia were in a state of hydropic degeneration and many of the more severely affected cells were grossly swollen. As the cell contents were clear and unstained and the nuclei were pressed to the periphery, these cells had a signet ring appearance. In areas where this was most marked the myelin sheaths of the nerve fibres were also considerably swollen in an irregular fashion and presented a beaded appearance (Fig. 2). The result was a patch of vacuolations consisting of both degenerating oligodendroglia and myelin. Vacuolation was also present near and around the capillaries. Despite the well-defined focal changes affecting glia and myelin there was remarkably little damage to nerve cells in the cerebrum. In the quadrate bodies there was an indication of commencing degenerative change in some of the neurons as was shown by a loss of cytoplasm, margination of nuclear material and mild satellitism. A diffuse microgliosis was present.

The cerebellum was mildly congested and although a diffuse gliosis was evident, it was less severe than in the anterior parts of the brain. There was a little round cell infiltrate, near the pial vessels, in the sulci of the cerebellum. There was no sign of neuronal damage. The reduction in severity of the changes continued into the medulla where there was mild congestion and a possible slight perivascular oedema. Gliosis was absent and the nerve cells appeared normal.

*Lambs 3 and 4.* The changes were of the same nature as in the two previous brains, but somewhat milder. The vacuolation of the forebrain was less in evidence and although the diffuse gliosis was still well marked the glial foci were not so large. There were small clumps of infiltrating cells, mainly of the small mononuclear type, near some of the blood vessels, but there was no suggestion of perivascular cuffing. Mild gliosis and congestion were present in the quadrate bodies. The remainder of the brain examined seemed normal.

*Lamb 5.* The only changes observed were in the cerebrum and they consisted of a mild diffuse gliosis and a few small glial foci in the white matter. Neither oligodendroglia nor myelin was vacuolated.

*Lambs 6 and 7.* No significant changes were present. The forebrain and meninges were, if anything, congested.

*Liver*

*Lambs 1 and 2.* Many hepatic cells of the central zones of the lobules were necrotic, the nucleus having lysed completely. In some places the necrosis extended into the mid-zone. The central

vein and sinusoids in the necrotic part contained a non-cellular but slightly granular eosinophilic fluid (Fig. 3). There was a hyperaemic belt at the margin of the zone of necrosis. When structure was not lost, most of the cells contained one or more intracytoplasmic, refractile, rounded bodies which gave a positive reaction for fat with Sudan IV. As the fat globules were not always clear in haematoxylin and eosin stained sections, they sometimes appeared like incomplete vacuoles in the liver cells. In one of the livers small infiltrations of a mixed cell type extended a short distance into the parenchyma from the portal tract. The biliary epithelium was swollen.

*Lambs 3 and 4.* Centrilobular necrosis was widespread in the organ. In some lobules the degenerative process extended well into the mid-zones whereas in others it was confined to the centre. The fat globules were scanty, but when present were in cells near the necrotic zone. Hyperaemia was occasionally severe. A feature of the damage was the large amount of cellular debris and granular eosinophilic fluid in the central zone. The infiltrating cells which were observed in the liver of lamb 1 were also present in cases 3 and 4 and although they were not numerous they were scattered throughout the parenchyma.

*Lamb 5.* In this liver some of the central zones had escaped necrosis while others which had been affected showed the earliest signs of repair manifested by regenerating liver cells and the presence of a very few fibrocytes. A leucocytic infiltration was present in all zones. The intracytoplasmic fat droplets were very infrequent. P.A.S. positive material could be found in the form of granules in all of the peripheral cells and in some of the mid-zone cells, but none was seen in the central cells. The granules were shown to be glycogen. The livers of the earlier cases failed to show this reaction.

*Lamb 6.* The liver was congested and mild cloudy swelling was widespread. A few irregularly-shaped fat droplets were present. Centrally some fibrocytes were active and the first, most delicate, collagen threads were made visible by van Gieson's method. Glycogen was distributed in the lobules as in the previous case.

*Lamb 7.* Little necrosis was evident, but a few delicate threads of collagen were present centrally in the lobules. The liver was rather congested. Both in this and the previous case there was a slight increase in connective tissue elements in the portal tracts.

*Kidney*

*Lambs 1, 2, 3 and 4.* In the renal cortex there was severe congestion of the larger blood vessels and some haemorrhage. The renal corpuscles were slightly congested. Only a few showed any degenerative change and they had lost small groups of cells into the lumen of Bowman's capsule. The cortical tubular lesions were more marked

and varied from severe cloudy swelling with luminal occlusion to fatty degeneration and on to frank necrosis. The cortico-medullary junction was congested and haemorrhages were present in the interstitial tissues and also in the uriniferous tubules. In most cases in this area the tubular epithelium was necrotic and lay in the lumen as masses of cellular debris. The degenerative changes continued, with less severity, through the medulla to the renal pelvis.

*Lambs 3 and 4.* The degree of congestion seemed less than in the two lambs which died earlier.

*Lamb 5.* In the cortex a slight distortion of some of the renal corpuscles had occurred. A variety of changes were present in the tubules. In many of them the epithelial cells were tremendously swollen and the cytoplasm remained unstained (Fig. 4). The nuclei retained their affinity for stain sufficiently to indicate that margination of chromatin had taken place. The picture was of parallel rows of large, clear, rounded balloon-like structures. Although this lesion was present at all levels of the kidney it was most in evidence in the convoluted tubules and the loop of Henle. It could be regarded as a severe form of hydropic degeneration, but in many of the affected cells fat was demonstrable. Elsewhere the epithelium had been desquamated and the tubules had collapsed. Other tubules, some of which also had lost their epithelium, were dilated to form elongated cysts. Some tubules were undamaged and in others the epithelium was slightly infiltrated with fat.

Haemorrhages were present into and around the tubules especially in the cortico-medullary junction, but they were not as severe as in the kidneys from the earlier cases.

*Lambs 6 and 7.* The main features were the marked hydropic or fatty degeneration of the epithelium of many of the tubules and a reduction in the amount of congestion and haemorrhage. Although necrosis was widespread much of the debris had been removed.

*Lung*

It is unnecessary to give a separate description of the changes in each case, as, with the exception of lamb 5, the pulmonary lesions were alike and consisted of patches of acute congestion of the alveolar (mural) capillaries together with an inpouring of blood into many of the alveoli. In some alveoli the fluid, either haemorrhagic or infrequently oedematous, contained a variety of cells including a few epithelioids. When present, the cellular infiltration whether peribronchiolar or alveolar, was very slight. Emphysema had ruptured the walls of some alveoli near the congested areas. Many of the bronchi contained desquamated epithelial cells mixed with other debris and eosinophilic fluid to form a plug in the lumen. The process was that of severe pulmonary congestion with excessive secretion of fluid and was of very recent development (Fig. 5).

Lamb 5 also had pulmonary lesions, but in this case the acute

congestive phase was over and the phase of leucocytic activity was marked. The lesion was a lobular pneumonia affecting the alveolar walls more than the bronchi and it appeared to be of about 3 or 4 days duration whereas the lungs from the other lambs showed lesions which were probably of only a few hours standing.

*Spleen*

In general there was reduced cellularity of the germinal centres. The erythrocyte content was markedly less than normal except in lamb 5. The apparent splenic ischaemia in the other case was probably related to the extremely congested state of the lung, the erythrocyte reserves having been transferred from the spleen to the depleted circulation. In lamb 5 the pulmonary congestion was relatively slight and the spleen appeared to contain a normal amount of blood. The lymphoid centres in this case were considerably reduced in area and in density of cellularity.

## DISCUSSION

In the brain the changes are most marked in the cerebrum and least in the medulla, i.e., the degree of severity lessening from fore to hind brain. In the lambs which succumb earliest the cerebral lesions are severe while the lambs which lived longest show none. This would indicate the reversibility of the brain lesion and its possible insignificance in relation both to the time of death and to the clinical signs observed, e.g. lamb 7 exhibited terminal convulsions, but lesions were not seen in those parts of the brain which were examined. It is known that the nervous signs observed in aldrin poisoning are associated with dysfunction of the autonomic nervous system (Gowdey and Stavraky, 1955) and that the brains of sheep poisoned by the closely related dieldrin may not show lesions (Jolly, 1954). However, following aldrin and dieldrin poisoning, brain lesions have been seen in other species, e.g., the dog (Kitselman, 1953) and the fowl ((Löliger, 1956). The present writer has also seen brain lesions in poultry following accidental poisoning of this kind (unpublished observations, 1957).

In the series of livers examined the acute toxic hepatitis is most marked about the fifth day after poisoning and this is followed by a healing process. Centrally the necrotic tissue is replaced by regenerated hepatic cells and there is also an indication of fibrous replacement which first appears at the ninth day. Garner (1957) points to the lack of reports of cirrhosis of the liver following poisoning with the group of pesticides to which aldrin belongs. While gross cirrhosis is an unlikely sequel, the present observations show that in the repair process a delicate deposition of fibrous connective tissue occurs centrally and also interlobularly. The healing process is associated with a resumption of normal function of the organ, so far as could be detected by the histological methods used, as the fatty change is

overcome and glycogen storage is resumed from about the seventh day after poisoning.

From first to last the kidney lesion is a very severe acute tubular nephritis (nephrosis) mainly of those parts of the tubules which lie in the cortex, but by no means confined to them. The glomerular lesions are relatively insignificant. The tubular changes include cloudy swelling, fatty infiltration, hydropic degeneration and necrosis of the epithelium. Unlike the liver there is no good evidence of a repair process in the kidneys of the present cases other than the removal of debris. The nephritis is, however, reversible under some circumstances. Kitselman (1953) states that in the case of dogs given sub-lethal levels of aldrin in the diet, the kidney lesion disappears after administration of the toxic agent ceases.

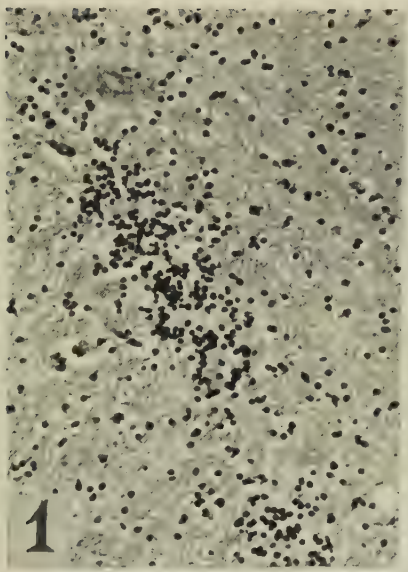
Of the direct adverse structural changes which occur immediately following aldrin poisoning in lambs it appears that tubular nephritis is the most important. In the present series of cases the brain and liver exhibit early and apparently successful attempts at repair, but the renal lesions remain very severe for 15 days, i.e., the survival time of the last lamb to die. It may be argued that the liver and brain are initially but little affected in the cases which survive longest. In the writer's opinion this is not so as the changes in the livers of the later cases are indicative of a repair process and in fact the brain lesions affect supporting rather than neural elements and are thus capable of early resolution. It is not denied, of course, that in all probability the lesions in those lambs which survived longest were originally the least severe.

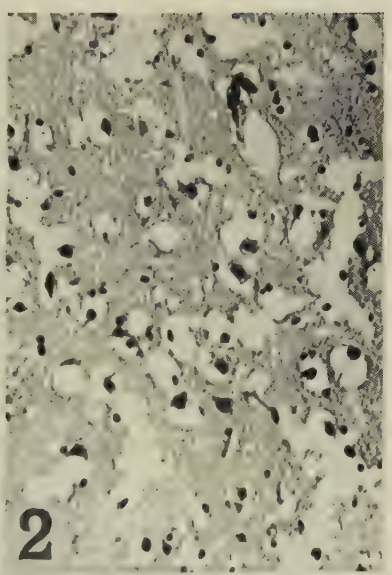
In contrast to the early onset and varying degree of persistance of the brain, liver and kidney changes attention may be drawn to the nature of the lung lesions which, with the exception of one case, indicate a very recent circulatory disturbance. A number of workers have indicated that death should be ascribed to this acute respiratory failure.

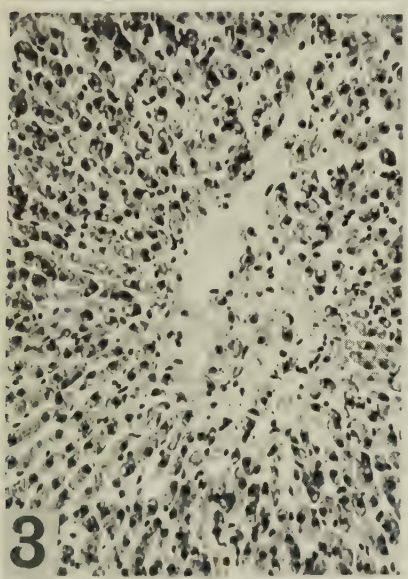
## CONCLUSIONS

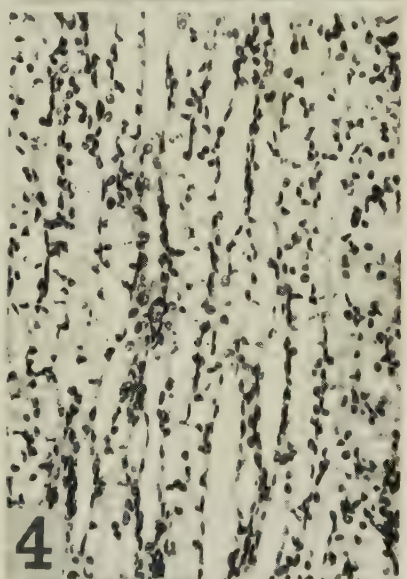
The histopathological changes observed in a series of 7 lambs which died at intervals of up to 15 days following accidental poisoning with aldrin are described.

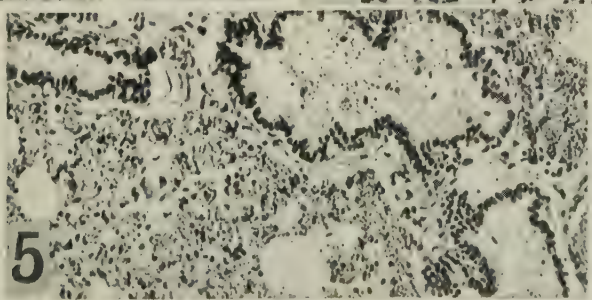
Reversible changes consisting mainly of diffuse and focal gliosis and focal degeneration of oligodendroglia and myelin were present in the fore brain. Neuronal damage was slight. The liver lesion was an acute toxic hepatitis in which a well marked central necrosis was seen to undergo a process of repair by regeneration of liver cells and to a minor degree by replacement with fibrous tissue. In the kidney there was an acute tubular nephritis which appeared to be the most important of the original lesions. There was no clear indication, in the sections examined, that the renal lesion was undergoing resolution.




*To face page* 190

The terminal lesion was acute pulmonary congestion associated with respiratory failure.

ACKNOWLEDGMENTS

I am indebted to colleagues for their co-operation, to Mr. S. P. Rodgers for technical assistance and to Mr. D. Mehaffey, who prepared the photomicrographs.

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[*Received for publication, July 14th,* 1958]

LEGENDS TO ILLUSTRATIONS

Fig. 1. Lamb 1. Glial foci in cerebrum.
Fig. 2. Lamb 2. Vacuolation in cerebrum.
Fig. 3. Lamb 2. Central necrosis and widespread fatty change of liver.
Fig. 4. Lamb 5. Severe degenerative changes of renal tubular epithelium.
Fig. 5. Lamb 7. Acute pulmonary congestion and intra-alveolar haemorrhage. Loose plug of debris in bronchiole.

All sections stained H. & E. Magnification × 125.

# THE EFFECTS OF DIFFERENT ANTICOCCIDIAL AGENTS ON THE INTESTINAL COCCIDIOSES OF THE FOWL

By

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## INTRODUCTION

The incidence of intestinal coccidiosis caused by *Eimeria necatrix* and *Eimeria acervulina* is increasing under conditions of intensive husbandry. Little has been published in Britain on the diseases produced by these species, or on their control by chemical means.

A redescription of the life history of *E. necatrix* was published by Davies (1956) and included a brief consideration of the treatment of the associated disease with sulphadimidine. Peterson and Hymas (1950) investigated the prophylactic effect of sulphaquinoxaline, 5-nitro-2-furaldehyde semicarbazone (nitrofurazone) and m, m'-dinitrodiphenyl disulphide (nitrophenide) on experimentally induced *E. necatrix* infestations in young chickens. They found that 0·01 per cent sulphaquinoxaline in water had little influence upon the course of the experimental disease when treatment was started twenty-four hours after the oocysts were administered and was continued for five or six days. At higher concentrations (0·02 per cent and 0·03 per cent) deaths were averted and the clinical nature of the disease was moderated. Nitrofurazone, administered in the feed at concentrations of 0·005 per cent to 0·03 per cent from the time of infection, significantly reduced the number of deaths and modified the course of the disease. Nitrophenide, at a concentration of 0·02 per cent in the feed, had little effect upon the course of the infection. Higher concentrations, 0·03 and 0·04 per cent, reduced the number of deaths. A still higher concentration, 0·05 per cent, completely suppressed mortality. Peterson and Munro (1949) considered that continuous low level medication with sulphaquinoxaline might prove of substantial benefit in controlling outbreaks of *E. acervulina*. Waletzky, Hughes and Brandt (1949) found that nitrophenide was active in controlling caecal and intestinal (*E. necatrix*) coccidiosis. Cuckler, Malanga and Ott (1956), working with the chemical complex formed by 4, 4'-dinitrocarbanilide and 2-hydroxy-4, 6-dimethylpyrimidine (nicarbazin), found that it prevented deaths which might arise from experimentally-induced infections of *E. necatrix* and that it could also be used with advantage in the control of *E. acervulina*.

As the available data relating to intestinal coccidiosis and its control in Britain are inadequate, it was decided to run some tests on a comparative basis to determine the most effective drugs for prophylaxis and for the treatment of established intestinal infections. The present paper reports the results of these tests.

## MATERIALS AND METHODS

White Leghorns (WL), Rhode Island Red (R.I.R.) and Rhode x White Leghorns (R x WL) chicks aged from 4 to 24 days were used. The chicks were reared in wire floor brooders in which they were kept until

required for experiment, when they were moved to tier brooders in the experimental house or, in a few cases, to wire-floored metal cages in the same house. The chicks were fed on mash of the formula detailed by Horton-Smith and Long (1956).

*Preparation of medicated foods.* The diets were freshly prepared at the outset of each experiment. Proprietary premixes were used as far as possible. The food was made up in 2,500 or 5,000 gram lots of dry mash. The premixes were incorporated in the mash by mixing in an electrically-driven earthed "Hobart" food mixer for 20 to 30 minutes.

*Preparation of medicated solutions.* The solutions were prepared each day in tap water following the manufacturers' instructions and were stored in dark glass bottles away from direct sunlight.

*Infective material.* Oocysts of *E. necatrix* were derived from a single cell isolated from a natural infection occurring in November 1956. A stock suspension was maintained under conditions of strict isolation. Cultures of sporulated oocysts were stored in 2·5 per cent potassium dichromate in the dark at room temperature in conical flasks lightly plugged with cotton wool. Tests have shown that oocysts stored in this way are infective for at least thirty-five days. The infective doses varied from 80,000 to 250,000 oocysts per chicken. The most commonly used dose was 100,000 oocysts. Oocysts of *E. acervulina* and *E. maxima* were obtained from field cases in December 1956 and September 1956 respectively. Experimental infections with these species were obtained with oocysts derived from single cell inocula. Doses for oocyst production were varied.

*Enumeration of oocysts in culture.* Counts were made in a Fuchs-Rosenthal counting chamber having a depth of 0·2 mm. and large squares of 1 sq. mm. By counting the oocysts in five squares in each of two chambers a total volume of 2 cu. mm. was examined. The oocyst culture was counted without dilution so that to obtain the number of oocysts per ml. the total number of oocysts counted in a volume of 2 cu.mm. was multiplied by 500. The counts were repeated at different times on the same culture.

*Determination of oocyst-production.* A modified method was used for the estimation of oocyst-production of *E. necatrix*, *E. acervulina* and *E. maxima* in medicated chicks. The faecal output over 24 hour periods was examined. The method used was that described by Long and Rowell (1958).

*Collection of oocysts from faeces.* Care was taken to ensure that no contamination with oocysts of other species occurred. Glassware was sterilised in a hot air oven and cheese cloth used for the removal of coarse debris was discarded after use. The oocysts were then freed from faecal matter as far as possible by spinning in saturated salt solution in tubes covered by glass slips which were then rinsed in water to remove the surface film with oocysts after each spinning. The oocysts so collected were then concentrated by spinning the washed suspension in the centrifuge.

## RESULTS

### *E. NECATRIX*

#### *Continuous Treatment*

*The survival of chickens after the start of medication.* Agents of known

TABLE 1

THE EFFECT OF STARTING THE MEDICATION OF CHICKENS WITH DIFFERENT ANTICOCCIDIAL DRUGS BEFORE INFECTION WITH *E. NECATRIX*

| *No. of experiment* | *Breed of chicken* | *Deaths from* E. necatrix *infection* — *Treatment given* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *No medication* | *SQ 0·0125% (F)* | *SQ 0·05% (F)* | *SQ 0·05% (IV)* | *NP 0·0125% (F)* | *B1 (F)* | *B2 (IV)* | *NZ 0·01% (F)* | *NZ 0·01% (IV)* | *NC 0·0125% (F)* |
| 1 | WL | 18/20 | 14/20 | — | — | — | — | — | 1/19 | — | 0/19 |
| 2 | RXWL | 7/8 | — | 5/8 | — | — | 1/8 | — | 0/8 | — | 0/8 |
| 3 | RXWL | 9/10 | — | 7/10 | — | 2/10 | — | — | 0/10 | — | 0/10 |
| 4 | RXWL | 17/20 | — | 10/20 | — | — | — | — | 3/20 | — | 0/20 |
| 5 | RXWL | 10/10 | — | — | 9/10 | — | — | 3/10 | — | 2/10 | 4/10 |
| 6 | RXWL | 7/10 | — | — | — | — | — | — | 4/10 | — | 0/10 |
| 7 | RXWL | 7/8 | — | — | — | 5/8 | 5/8 | 2/8 | — | 1/8 | — |
| 8 | RXWL | 9/10 | — | — | — | — | — | 0/10 | — | — | — |
| 9 | WL | 7/7 | — | — | — | — | 3/9 | — | 6/9 | — | 0/9 |
| 10 | WL | 7/9 | — | — | — | 8/9 | 3/9 | 0/9 | 1/9 | — | — |
| Total mortality | | 98/112 | 14/20 | 22/38 | 9/10 | 15/27 | 12/34 | 5/37 | 15/85 | 3/18 | 4/86 |
| Per cent mortality | | 87·5 | 70·0 | 57·9 | 90·0 | 55·5 | 35·3 | 13·5 | 17·5 | 16·6 | 4·6 |

*B1* = 0·0055 per cent nitrofurazone + 0·0008 per cent furazolidone (Bifuran)
*B2* = 0·011 per cent nitrofurazone + 0·0016 per cent furazolidone (Bifuran)
*NC* = nicarbazin
*NZ* = nitrofurazone
*NP* = nitrophenide
*SQ* = sulphaquinoxaline
(*F*) = drug administered in the food
(*W*) = drug administered in the water.

activity against *E. tenella* were used either in the food or water and in most cases at the concentrations recommended by the manufacturers. Treatment started two days before the chicks were infected with oocysts and was continued for ten days in all. Survivors were killed seven to fourteen days after the last death had occurred. In this series of experiments the chickens were aged from 9 to 24 days at time of infection. They were infected with *E. necatrix* oocysts from cultures aged from 3 to 28 days.

The results given in Table 1 suggest that nicarbazin was the most efficient of the drugs tested. Bifuran (0·011 per cent nitrofurazone +0·0016 per cent furazolidone) in water (B2 in Table 1) and nitrofurazone in either food or water occupied second and third place respectively. Sulphaquinoxaline (at 0·05 per cent in the food) gave only about 42 per cent and nitrophenide (at 0·0125 per cent in the food) only about 45 per cent protection.

*The survival of chickens before the start of medication.* In this series of ten experiments, groups of chickens were infected with oocysts and two days later were given medicated food or water for five days. In most cases the surviving chickens were destroyed between the second and third week after receiving the oocysts. The ages of the chickens used in this series varied from 4 to 21 days and those of the *E. necatrix* cultures from 8 to 35 days.

When medication was delayed the concentration of sulphadimidine in the food had to be raised to 1·0 per cent w/w to give about 77 per cent protection. The recommended concentration of 0·2 per cent in the water gave only about 42 per cent protection. Sulphaquinoxaline at a concentration of 0·05 per cent in water protected rather less than half and Bifuran (0·011 per cent nitofurazone plus 0·0016 per cent furazolidone) in water (B2 in Table 2) approximately 83 per cent of the infected chickens. In another experiment, not included in Table 2, in which food containing 0·02 per cent nitrofurazone or Bifuran (0·011 per cent nitrofurazone +0·0016 per cent furazolidone) was offered to chicks 48 hours after infection, approximately 82 and 73 per cent protection was obtained. Nitrophenide also gave substantial protection when included in the food at a concentration of 0·05 per cent w/w. Furazolidone was not entirely satisfactory, but on taking into consideration the four experiments as a whole about 77 per cent protection was afforded.

*Intermittent Treatment*

This form of medication, using sulphadimidine and sulphaquinoxaline solutions, was applied to *E. necatrix* infections according to the treatment schedule given in Table 3. Table 4 summarises the effects. Sulphaquinoxaline, at a concentration of 0·064 per cent, gave the best protection when treatment was started one day before the administration of oocysts and covered the period of schizogony occurring on the 4th and 5th days of the infection (Group 2B).

TABLE 2

THE EFFECT OF DELAYING MEDICATION UNTIL THE SECOND DAY AFTER THE INFECTION OF CHICKENS WITH *E. NECATRIX*

| *No. of experiment* | *Breed of chickens* | *No. of oocysts in infective dose* | *Deaths from* E. necatrix *infection* — *Treatment given* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *No medication* | *SQ 0·1% (F)* | *SQ 0·05% (IV)* | *SD 1·0% (F)* | *SD 0·2% (IV)* | *NP 0·05% (F)* | *B1 (IV)* | *B2 (IV)* | *NZ 0·02% (F)* | *NZ 0·01% (IV)* | *NZ 0·02% (IV)* | *FUR 0·04% (F)* |
| 1 | WL | 100,000 | 14/15 | — | — | 2/16 | — | — | — | — | 2/16 | — | — | — |
| 2 | RXWL | 250,000 | 8/10 | 4/10 | 4/10 | 4/10 | 4/10 | — | — | — | 0/10 | — | — | — |
| 3 | RXWL | 80,000 | 9/10 | — | 4/10 | — | 5/10 | — | — | — | — | 3/10 | — | — |
| 4 | RXWL | 100,000 | 17/20 | — | 6/10 | — | 9/10 | — | 2/10 | — | — | 1/10 | — | — |
| 5 | RXWL | 100,000 | 10/10 | — | 7/10 | — | 5/10 | — | 3/10 | — | — | 3/10 | — | — |
| 6 | RXWL | 100,000 | 7/10 | — | — | — | — | — | — | 1/10 | — | — | 2/10 | 6/10 |
| 7 | RXWL | 100,000 | 7/8 | — | — | — | — | — | — | 1/8 | — | — | 2/8 | 0/8 |
| 8 | RXWL | 100,000 | 9/10 | — | — | — | — | 3/9 | — | 4/9 | — | — | 2/9 | 2/9 |
| 9 | RXWL | 100,000 | 7/7 | — | — | — | — | 0/8 | — | — | 2/8 | — | — | 0/8 |
| 10 | WL | 100,000 | 7/9 | — | — | — | — | — | — | 0/9 | — | — | 0/9 | — |
| Total mortality .. .. | | | 95/109 | 4/10 | 21/40 | 6/26 | 23/40 | 3/17 | 5/20 | 6/36 | 4/34 | 7/30 | 6/36 | 8/35 |
| Per cent mortality .. .. | | | 87·1 | 40·0 | 52·5 | 23·0 | 57·5 | 16·8 | 25·0 | 16·6 | 11·7 | 23·3 | 16·6 | 22·8 |

*FUR*=furazolidone. *SD*=sulphadimidine. Other abbreviations as in Table 1.

Sulphadimidine, at a concentration of 0·1 per cent, and used in the same way (Group 2) had little or no effect on the course of infection.

*Effect of Different Drugs on Oocyst-production*

Five groups of six 3 week old R x W.L. chickens were given food medicated with 0·05 per cent sulphaquinoxaline, 0·0125 per cent nicarbazin, 0·01 per cent nitrofurazone, 0·0125 per cent nitrophenide and no medication respectively. One day after the beginning of medication, which was continued for twelve days in all, each chicken was given 2000 sporulated oocysts of *E. necatrix*. The approximate oocyst-production per chicken in these groups over the sixth to tenth days of the infection was as follows: sulphaquinoxaline 2,000,000, nicarbazin 0, nitrofurazone 41,800, nitrophenide 953,000 and the unmedicated group 2,000,000. The highest oocyst-production per chicken occurred between the ninth and tenth days in the chickens of the sulphaquinoxaline group, between the eight and ninth days in the nitrofurazone and nitrophenide groups and between the seventh and eighth days in the unmedicated group. When the over-all oocyst-production for the sixth to the tenth day period is considered there are marked differences between the oocyst-production in the nicarbazin and nitrofurazone-treated chickens on the one hand and that of the sulphaquinoxaline-treated and unmedicated chickens on the other. There is little difference in oocyst-production between the sulphaquinoxaline-treated and unmedicated chickens. The later appearance of maximum oocyst-production in the sulphaquinoxaline group suggests a small effect. The oocyst-production in the nitrophenide-treated chickens occupies an intermediate position between the two extremes. The possibility that a rise in oocyst-production in all groups might occur after the withdrawal of treatment cannot be ignored. By using mortality and oocyst-production as the criteria for estimating the efficiency of an anticoccidial agent, nicarbazin is obviously the most active of the agents tested.

*E. ACERVULINA*

As *E. acervulina* does not usually cause death of chickens the effect of various drugs, apart from autopsy, can best be assessed by using their influence on oocyst-production as a criterion of their anticoccidial activity. Three groups each of fifteen 1 week old W.L. chickens were treated as follows: one group received food containing 0·01 per cent nitrofurazone, one group received food containing 0·0125 per cent nicarbazin and the third group received food containing 0·0125 per cent sulphaquinoxaline. A further group of thirteen chickens received unmedicated food. Each chicken was infected with 500,000 sporulated oocysts on the day after medication was started.

Two chickens in the unmedicated group died, one on the sixth day and the other on the seventh day of the infection. Examination

TABLE 3

SCHEDULE OF TREATMENT

| *Group* | | *Sulphadimidine* | *Sulphaquinoxaline* | *Days in relation to infection with oocysts* | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *SD* | *SQ* | *Percentage in water* | | *−2* | *−1* | *0* | *+1* | *+2* | *+3* | *+4* | *+5* | *+6* | *+7* | *+8* | *+9* | *+10* | *+11* | *+12* | *+13* | *+14* | *+15* | *+16* |
| 1 | | 0·2 | | T | T | T | | | T | T | T | | | T | T | T | | | | | | |
| | 2A | | 0·032 | | | | | | | | | | | | | | | | | | | |
| 2 | 2B | 0·1 | 0·064 | | T | T | T | | | T | T | T | | | T | T | T | | | | | |
| 3 | | 0·2 | | | | T | T | T | | | T | T | T | | | T | T | T | | | | |
| | 4A | | 0·032 | | | | | | | | | | | | | | | | | | | |
| 4 | | 0·1 | | | | | T | T | T | | | T | T | T | | | T | T | T | | | | |
| 5 | | 0·2 | | | | | | T | T | T | | | T | T | T | | | T | T | T | | |
| | 6A | | 0·032 | | | | | | | | | | | | | | | | | | | |
| 6 | 6B | 0·1 | 0·064 | | | | | | T | T | T | | | T | T | T | | | T | T | T | |
| | 7A | | 0·032 | | | | | | | | | | | | | | | | | | | |
| 7 | | 0·1 | | | | | | | | T | T | T | | | T | T | T | | | T | T | T |

T = treatment *SD* = sulphadimidine *SQ* = sulphaquinoxaline

The results obtained with these forms of intermittent treatment are given in Table 4

of the intestines of these chickens revealed a greyish mucoid exudate in the duodenum and small intestine and large numbers of coccidial stages. White spots were seen on the serous surfaces of the duodenum and some haemorrhagic splashes were present. A similar, but more pronounced, pathological picture was later obtained by giving a single chicken 40,000,000 sporulated oocysts in each of two doses administered on successive days. The chicken died on the sixth day of the infection. The approximate output of oocysts per surviving chicken between the fourth and the thirteenth days of infection in the nicarbazin group was 1,400,000, in the nitrofurazone group 29,600,000, in the sulphaquinoxaline group 2,900 and in the unmedicated group 42,000,000. All the drugs substantially reduced oocyst-production, but sulphaquinoxaline reduced it to relatively small proportions. Some caution, however, is necessary in interpreting these figures since the difference is not very marked when the variation between groups within these treatments is considered.

TABLE 4

THE EFFECT OF INTERMITTENT TREATMENT WITH SULPHADIMIDINE AND SULPHAQUINOXALINE STARTED BEFORE AND AFTER THE TIME OF ADMINISTRATION OF *E. NECATRIX*

| *Groups* | | *Deaths from* E. necatrix *infection* | | | | | | | | | | *Total deaths* | *Number of chicks in group* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Days after infection with oocysts* | | | | | | | | | | | |
| *SD* | *SQ* | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *13* | *14* | | |
| 1 | | 4 | 1 | 1 | 1 | | | | | | | 7 | 10 |
| 2 | | 6 | 2 | | | 1 | | | | | | 9 | 10 |
| | 2A | 6 | 2 | 1 | | | | | | | | 9 | 10 |
| | 2B | 2 | | 1 | | | | | | | | 3 | 10 |
| 3 | | 1 | 7 | | | | | | | | | 8 | 10 |
| 4 | | 5 | | 3 | | | | | | | | 8 | 10 |
| | 4A | 8 | 1 | | | | | | 1 | | | 10 | 10 |
| 5 | | 3 | 3 | | | | | | | | | 6 | 10 |
| 6 | | 4 | 6 | 1 | | | | | | | | 11 | 11 |
| | 6A | 6 | 4 | | | | | | | 1 | | 11 | 11 |
| | 6B | 7 | 1 | | | | | | | | | 8 | 11 |
| 7 | | 3 | 8 | | | | | | | | | 11 | 11 |
| | 7A | 5 | 4 | | | | | | | | | 9 | 11 |
| No medication | | 6 | 1 | 2 | | | | | | | | 9 | 10 |

Details of treatment—See Table 3.

As sulphaquinoxaline treatment substantially reduced the oocyst-production of *E. acervulina* in the preceding experiment a second test was performed using two strengths of sodium sulphaquinoxaline. To observe the effect of delayed treatment medication was not started until late in the life-cycle, i.e. on the fourth day of infection, and was continued for ten days. Eighteen 3 week old R x WL chickens each received 500,000 sporulated oocysts of *E. acervulina* and were then divided into three groups of six chickens each. One group was treated with 0·0125 per cent and a second group with 0.025 per cent sulphaquinoxaline in the drinking water. Treatment started on the fourth day of infection and continued for ten days. The third group received no medication. The faeces were examined for oocysts each day from the third to fourth day until the sixteenth-seventeenth days of the infection. The details of the counts are shown in Table 5.

Schizonts were already present when treatment started as is shown by the presence of merozoites in the faeces on the third to fourth day. Despite two days' treatment, the counts obtained in all groups on the fourth-sixth days were high but there was some reduction in numbers in the two treated groups as compared with the controls. This reduction became more marked as the test continued.

The effect of three anticoccidial agents on infestation with *E. acervulina* was studied in a single experiment using very large numbers of sporulated oocysts administered in four doses on successive days to determine the effects of the drugs on mortality, oocyst-production and weight gain.

Fifty 8 days old R x WL. chickens were divided into five groups of ten. Four groups received medication as follows:—0·125 per cent sulphaquinoxaline, 0·05 per cent sulphaquinoxaline 0·01 per cent nitrofurazone and 0·0125 per cent nicarbazin in the food respectively. The fifth group received unmedicated food. Treatment started 24 hours before each chicken received its first infective dose of 10,000,000 oocysts and was continued for fourteen days. The initial infective doses were followed by three doses of 5,000,000, 3,000,000, and 2,000,000 oocysts given on three consecutive days. The faeces of the surviving chickens were examined for oocysts. The relative numbers were roughly estimated by removing the surface films of the salt solutions from the centrifuge tubes, viewing them under the low power of the microscope and noting the extent to which the field was populated.

A high death rate from intestinal coccidiosis caused by *E. acervulina* is not to be expected and it is only rarely that numbers of deaths occur on a single day. The principal effect on the chicken of a heavy infection with this species is a retarded growth rate. The mortality resulting from these multiple infections in treated and untreated chickens is given in Table 6 together with the relative oocyst-production of survivors. Treatment must be started before

TABLE 5

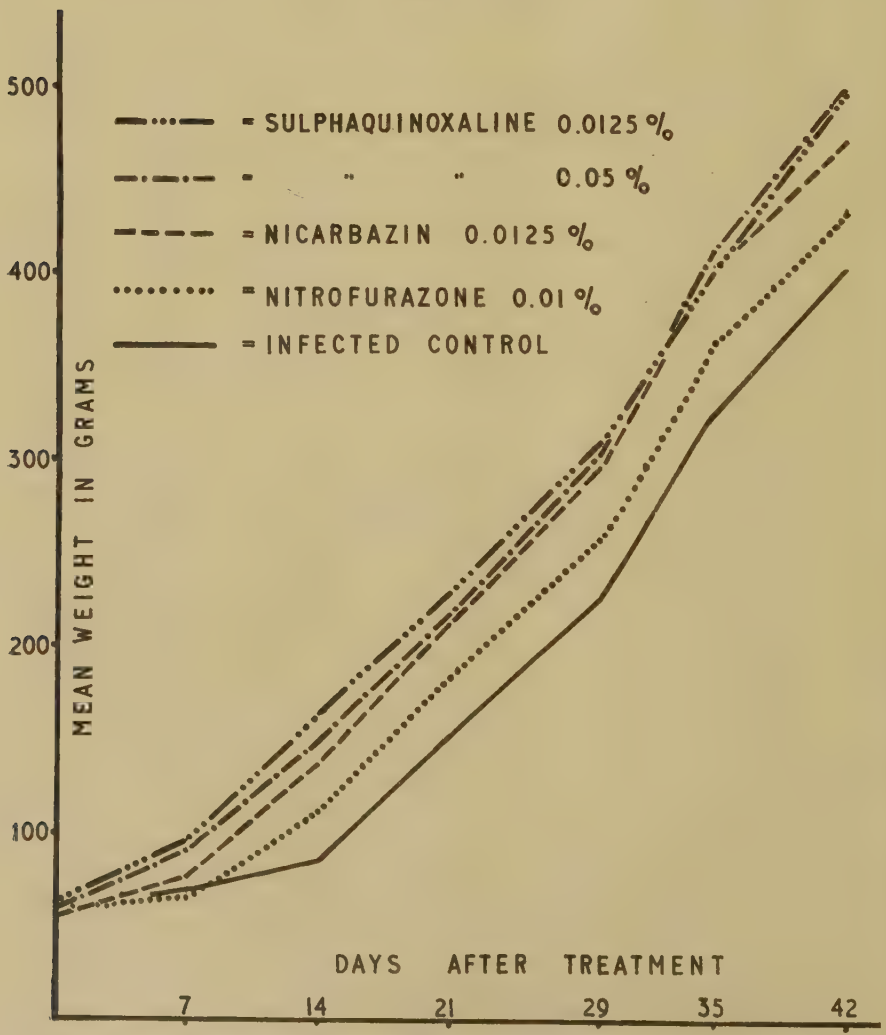
EFFECT OF SODIUM SULPHAQUINOXALINE SOLUTIONS ON THE OOCYST PRODUCTION OF *E. ACERVULINA*

| *Days after infection* | *SQ 0·0125% 4 days after infection for 10 days* | *SQ 0·025% 4 days after infection for 10 days* | *Infected control—no medication* |
|---|---|---|---|
| 3–4 | Merozoites but no oocysts present | Merozoites but no oocysts present | Merozoites but no oocysts present |
| 4–6 | 190,030,000 | 71,800,000 | 794,160,000 |
| 6–7 | 8,616,000 | 783,000 | 276,600,000 |
| 7–8 | 933,000 | 383,000 | 188,160,000 |
| 8–9 | 240,000 | 222,000 | 195,060,000 |
| 9–10 | 100,000 | 77,300 | 101,660,000 |
| 10–11 | 44,000 | — | 26,773,000 |
| Total 3–11 days | 199,963,000 | 73,265,300 | 1,582,413,000 |
| 11–13 | Few oocysts present | Few oocysts present | Few oocysts present |
| 13–14 (end of treatment) | ,, ,, ,, | ,, ,, ,, | ,, ,, ,, |
| 14–15 | ,, ,, ,, | ,, ,, ,, | ,, ,, ,, |
| 15–16 | No oocysts present | No oocysts present | ,, ,, ,, |
| 16–17 | ,, ,, ,, | ,, ,, ,, | ,, ,, ,, |

the infection is initiated if the maximum effect produced by sulphaquinoxaline is to be obtained.

The effect of treatment on the weight gains of the surviving chickens is shown in Fig. 1. The evidence obtained from mortality, oocyst-production and weight gains shows the superiority of sulphaquinoxaline as an anticoccidial agent against *E. acervulina*. Although the number of chickens available did not permit the inclusion of treated, uninfected groups or a group of untreated and uninfected chickens, it is felt that the data given in the figure do suggest that the treatments modified the course of the infections.

Fig. 1




The effect of feeding food medicated with sulphaquinoxaline, nicarbazin and nitrofurazone on the weight gain of chickens infected with *E. acervulina*.

TABLE 6

OOCYST-PRODUCTION AND MORTALITY IN CHICKENS RECEIVING TREATMENT STARTING 24 HOURS BEFORE INFECTION WITH *E. ACERVULINA*

| *Medication percentage in food* | *No. of chickens* | *Days after first infection with 10,000,000 oocysts* | | | | | | | | | | | | | *Deaths from* E. acervulina *infection* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *13* | *14* | *15* | *16* | *17* | |
| 0·0125 SQ | 10 | — | — | — | — | — | — | — | — | — | — | — | — | (++) | 0 |
| 0·05 SQ | 10 | — | — | — | — | — | — | — | — | — | — | — | — | (++) | 0 |
| 0·01 NZ | 10 | — | 1 (++) | 1 (++++) | (+++) | (++) | (++) | (++) | 1 (++) | (++) | (++) | (+) | (+) | (+) | 3 |
| 0·0125 NC | 10 | 1 | F | F | (++) | (+) | (+) | (+) | (+) | 1 (+) | (+) | (+) | (+) | (+) | 2 |
| No treatment | 10 | 1 | (++) | 1 (++++) | (++++) | (++++) | (++++) | (++++) | 1 (++++) | (++++) | 1 (++++) | (+++) | (++) | (+) | 4 |

(++++)=very large numbers (+++)=large numbers (++)=moderate numbers (+)=small numbers F=few
SQ=sulphaquinoxaline NZ=nitrofurazone NC=nicarbazin

TABLE 7

OOCYST-PRODUCTION IN CHICKENS RECEIVING TREATMENT STARTING 24 HOURS BEFORE INFECTION WITH *E. MAXIMA*

| *Days after infection* | *Treatment* | | | |
|---|---|---|---|---|
| | *Nitrofurazone at 0·01 per cent in food* | *Sulphadimidine at 0·2 per cent in water* | *Nicarbazin at 0·0125 per cent in food* | *No medication* |
| 5–6 | 0 | 0 | 693,200 | 1,573,300 |
| 6–7 | 301,500 | 0 (T) | 6,671,300 | 43,322,600 |
| 7–8 | 53,300 (T) | 0 | 3,030,900 (T) | 10,133,000 |
| 8–9 | 8,500 | 0 | 363,000 | 53,000 |
| 9–11 | Flotation negative | Few on flotation | Few on flotation | Flotation negative |
| Total oocysts obtained by count | 363,300 | 0 | 10,758,400 | 55,081,900 |

(T) = end of treatment.

### *E. MAXIMA*

Deaths seldom occur as a consequence of *E. maxima* infestations and what has been said about *E. acervulina* applies in large part to this species as well. Three groups, each of six 3 weeks old RIR chickens, were treated with (1) 0·01 per cent nitrofurazone for nine days (2) 0·2 per cent sulphadimidine on a schedule of interrupted treatment of two three-day periods of medication separated by two days non-medication and (3) 0·0125 per cent nicarbazin for nine days. A further group received no medication. The chickens were each infected with 10,000 sporulated oocysts twenty-four hours after medication had started in the relevant groups. Counts of oocysts were made from the fifth to sixth day to the eleventh day of infection. The results are given in Table 7.

A variation in susceptibility of individual chickens to *E. maxima* infections became evident during this work but, on the whole, nitrofurazone and sulphadimidine exerted considerable control on oocyst-production; sulphadimidine almost completely suppressed oocyst-production up to the eleventh day of the infection and there seems to be little doubt that anticoccidial sulphonamides are superior to other agents. Although nicarbazin substantially reduced the number of oocysts produced, it did not bear any comparison with the other drugs in its capacity to reduce oocyst-numbers.

## DISCUSSION

Sulphadimidine and sulphaquinoxaline proved less effective than nitrofurazone and nicarbazin in controlling infestations with *E. necatrix*. In whatever way the sulphonamides were applied the results obtained were inferior to those obtained with the other drugs. It is just possible that the particular strain of *E. necatrix* used might have been partially resistant to sulphonamides at the time of isolation although there was no evidence to suggest this. Whether certain strains of coccidia are inherently more susceptible or more resistant than others to the action of a drug can only be answered by using a number of strains of different origin. Sulphadimidine afforded fair control of *E. necatrix* infestations when used prophylactically, but it is not suitable for use in long treatment in view of its reported toxicity. Sulphadimidine also gave substantial control when applied to established infestations, but only when the concentration was raised to a dangerously high level. The mixture of nitrofurazone and furazolidone (Bifuran) in soluble form and given at the higher strength offered satisfactory protection when used prophylactically or therapeutically, but nitrofurazone alone was almost as good. The best results were obtained with nicarbazin. *E. acervulina* and *E. maxima* did not show the same responses to these drugs for, whereas sulphaquinoxaline substantially reduced the oocyst-production of both species and the mortality arising from *E. acervulina*, nitrofurazone and nicarbazin failed to prevent deaths

from *E. acervulina* infections and only slightly reduced the output of oocysts of both species.

The investigation was undertaken to determine the relative anticoccidial properties of these drugs on species other than *E. tenella* and no account has been taken of possible toxicity although evidence of such effects exists. Reports from the United States suggest that glycarbylamide possesses the anticoccidial qualities of nicarbazin and is relatively non-toxic. Horton-Smith and Long (in preparation) have found this compound highly active against *E. tenella* and *E. necatrix*. In reviewing the results it appears that the different species of coccidia do not respond equally well to the same treatments. The best control can only be obtained when the specific cause of the disease is known and the appropriate drugs are selected.

It has been shown that 1.0 per cent w/w sulphadimidine in the diet protects a fair proportion of chickens from infections developing from inocula of 100,000 oocysts of *E. necatrix*. Subsequent studies have shown that the addition of 0.01 per cent p-aminobenzoic acid (PABA) to this medicated feed almost completely inhibits the anticoccidial effect of the sulphonamide. The mechanism by which sulphadimidine controls *E. necatrix* is the same as that by which it controls *E. tenella*, although the protective effect of sulphadimidine is not so marked at the recommended concentration. Potentiation tests, using mixtures of sulphadimidine and a pteridine compound in the diet, followed as a corollary to observations on the effect of PABA. *E. tenella* infestations were almost completely controlled by including a mixture of 0.05 per cent sulphadimidine and 0.005 per cent pteridine in the feed for three days from the second day of the infection. The same mixture failed to control infestations with *E. necatrix*. On the other hand, *E. necatrix* infestations were substantially controlled when 0.1 per cent sulphadimidine and 0.01 per cent pteridine were included in the diet for the same period. When the concentrations of sulphadimidine and pteridine were increased to 0.2 per cent and 0.02 per cent respectively, complete protection was obtained. Sulphadimidine alone (0.2 per cent) gave little or no protection. A sulphadimidine concentration of 1.0 per cent, and the combined sulphadimidine and pteridine concentrations used to obtain these results were experimental dosages and are not to be regarded as recommended procedures for the control of field outbreaks of coccidiosis.

In a single experiment in which *E. necatrix* infestations were reduced so as to give a 50 per cent mortality or less in the untreated control group, a course of intermittent treatment of three 3-day periods of treatment with 0.2 per cent sulphadimidine in the diet separated by two 2-day periods of no treatment, and starting on the second day of the infection, reduced the number of deaths. A detailed account of these experiments will be published later.

## CONCLUSIONS

The anticoccidial activities of sulphaquinoxaline, sulphadimidine, nitrofurazone, nicarbazin and the mixture of nitrofurazone and furazolidone (Bifuran) at two strengths were tested against experimentally induced infections with *E. necatrix, E. acervulina,* and *E. maxima.*

When treatment was started before infection the best protection against *E. necatrix* was given by nicarbazin, nitrofurazone and the higher strength of Bifuran.

Good protection against established infections with *E. necatrix* was obtained with the higher strengths of nitrophenide, nitrofurazone and Bifuran.

Sulphaquinoxaline protected chickens against heavy infections with *E. acervulina.* Sulphadimidine and nitrofurazone either completely suppressed or greatly reduced oocyst-production of *E. maxima.*

## ACKNOWLEDGMENTS

We are grateful to Messrs. May & Baker, Messrs. Smith, Kline & French, Messrs. Merck, Sharp & Dohme, Messrs. Cyanamid of Great Britain Limited and Messrs Allen and Hanbury Ltd., for supplying the compounds used in these tests.

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[*Received for publication, August 16th,* 1958]

# STAPHYLOCOCCAL ANTITOXIN IN THE BLOOD AND MILK OF CATTLE

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## INTRODUCTION

This investigation was carried out to gain information on the occurrence of staphylococcal antitoxin in the blood of farm animals, more especially of the milking cow. A further purpose was to study the antitoxin content of the milk and its relation to infection of the udder with staphylococci. The work is a sequel to the definition by Edwards and Rippon (1957) of some of the species of staphylococci pathogenic for the bovine udder.

The subject was studied in 1937 by Minett who demonstrated the presence of staphylococcal antitoxin in the blood of cattle, sheep and goats, and showed that in cattle there was a direct relationship between age and the antitoxin titre present in the blood. He also noted a rise in antitoxin level in the blood of naturally infected cows after calving and this was believed to be due to the increased activity of staphylococci in the udder at this time. Antitoxin was often found in milk whey, but no correlation could be established between the titre present and specific infection due to *Staph. aureus*. On the other hand, Lasmanis and Spencer (1954) found that 90 per cent of quarters infected with coagulase positive staphylococci had whey titres of 0·5 units per ml. or over, whereas only 14 per cent of non-infected quarters possessed similar titres. They also found a correlation between the pH of the milk and the antitoxin titre of the whey.

## MATERIALS AND METHODS

Examination of blood serum for the presence of staphylococcal antitoxin was carried out by a test which was similar in principle to that described by Minett (1937, 1939). Doubling dilutions of serum were titrated against an Lh dose of toxin determined by a preliminary test against standard antitoxin. For the production of $\alpha$ toxin, the Woods strain was grown in sloppy (0·3 per cent) agar for 4 days at 37°C in 20 per cent $CO_2$, and for $\beta$ toxin, strain 7428 (National Type Collection, Medical Research Council) was used. Standard $\alpha$ and $\beta$ antitoxins were kindly supplied respectively by the departments of Biological Standards of the Medical Research Council and of the State Serum Institute, Copenhagen, at a potency of 20 units per ml. For convenience in the test, $\alpha$ toxin was diluted 1 in 10 while the $\beta$ toxin was not diluted, and falling dilutions were titrated against 0·1 units of the standard antitoxins. The test as used in the earlier part of the work was capable of detecting 0·25 units of antitoxin, but by suitable dilution of toxin its sensitivity was increased to detect 0·025 units antitoxin per ml. Milk samples were tested in the same way as serum samples, and with a little practice the lysis or its absence could be readily distinguished in the presence of whole milk.

The presence of pathogenic organisms was detected by streaking a loopful of gravity cream on the surface of bacto-tryptose agar to which was added 5 per cent ox-blood of low antitoxin content. The alkalinity of the samples was determined by adding brom-cresol purple indicator to the sample.

## RESULTS

### *Antitoxin in the Blood*

*Occurrence.* The sera of 202 cows and heifers belonging to three herds at Compton were examined for the presence of α and β antitoxin. Of these, 159 (79 per cent) contained α antitoxin in a titre of 0·25 units/ml. or over and 119 (59 per cent) β antitoxin. Of 22 heifers between 1 and 2 years of age only 3 showed α antitoxin and none β antitoxin whereas of 40 in-calf heifers between 2 and 3 years of age 27 contained α antitoxin and 8 β antitoxin. Of 140 samples from cattle over this age only 11 showed no α and 29 no β antitoxin. It will be seen in Table 1 that the titre of antitoxin increased with age though there was considerable variation from the mean titre among animals in the same age group. While the rise in both α and β antitoxin was appreciable in cattle belonging to Herds 1 and 3, it will be seen that the rise in β antitoxin in cattle belonging to Herd 2 was by comparison small and that the final level was lower than that in cattle belonging to the other two herds. This difference in titres of β antitoxin is probably explained by the fact that the prevailing staphylococcus present in milk samples from Herd 2 was a phage type 3A (type 2 Edwards and Rippon, 1957) which does not produce β haemolysin in vitro. On the other hand, Herd 1 was infected with a 42D type and Herd 3 with a 78 type which produce both α and β haemolysins.

Milk samples taken at the same time as blood samples from cows in Herds 1 and 2 were found to contain pathogenic staphylococci in practically all cows in their second lactation, or later, and regular tests over many years have confirmed the high incidence of infection in these two herds.

*Titre at calving.* The blood of 25 cows was tested for the presence of antitoxin at 2 weeks and 1 week before calving, on the day of calving and again at 2-day intervals after calving until no antitoxin was detectable. It was observed that there was a rise in the antitoxin level occurring at 2 to 7 days after calving in 5 of these cows, of which two developed clinical mastitis due to pathogenic staphylococci. Blood samples from a further 52 cows were also examined at calving and up to 10 days after calving. In 4 cases, clinical mastitis due to staphylococci developed soon after calving and in 3 of these there was a simultaneous rise in the antitoxin titre of the blood.

Thus the evidence from these findings does not support Minett's observations that increases in antitoxin titre invariably occur after calving but point to the fact that such increases only take place when clinical mastitis due to staphylococcal infection ensues.

TABLE 1

STAPHYLOCOCCAL INFECTION OF THE UDDER AND ANTITOXIN TITRE OF THE BLOOD

mean antitoxin titre in units/ml.

| *Herd* | | *1 Year* α | β | *2 Years* α | β | *3 Years* α | β | *4 Years* α | β | *5 Years* α | β | *6 Years* α | β | *7 Years and over* α | β |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 (90 cattle) | No. tested | 22 | | 11 | | 10 | | 12 | | 13 | | 11 | | 11 | |
| | No. showing staphylococci in milk | — | | — | | 0 | | 8 | | 10 | | 8 | | 10 | |
| | Mean serum titres | <0·25 | — | 1·12 (±0·42) | <0·25 | 0·85 (±1·12) | 0·62 (±1·16) | 3·03 (±3·9) | 1·2 (±1·02) | 3·6 (±3·2) | 2·1 (±2·36) | 6·6 (±8·1) | 4·68 (±3·96) | 8·21 (±4·54) | 6·18 (±4·42) |
| 2 (61 cattle) | No. tested | 0 | | 13 | | 8 | | 12 | | 2 | | 15 | | 11 | |
| | No. showing staphylococci in milk | | | — | | 5 | | 9 | | 1 | | 13 | | 11 | |
| | Mean serum titres | | | 0·67 (±1·15) | <0·25 | 2·68 (±4·5) | 0·36 (±0·41) | 3·24 (±3·0) | <0·25 | 3·3 (±1·12) | 0·34 (±0·1) | 5·7 (±4·8) | 1·3 (±1·98) | 9·7 (±11·3) | 1·6 (±3·6) |
| 3 (51 cattle) | No. tested | 0 | | 16 | | 13 | | 5 | | 17 | | | | | |
| | Mean serum titres | | | 1·48 (±2·37) | 0·8 (±1·29) | 5·2 (±4·7) | 3·7 (±4·2) | 18·7 (±8·6) | 6·4 (±2·0) | 11·3 (±9·85) | 7 (±5·5) | | | | |

*Titre in newly born calves.* Minett (1937) showed that the serum of lambs born of ewes which had been injected with staphylococcal toxoid in the later stages of pregnancy contained antitoxin which was detectable for at least 40 days after birth. Samples of serum from 26 calves were examined for the presence of antitoxin before they had suckled. They were again tested at 2 days and 7 days of age in the case of bull calves, and up to 28 days in the case of heifer calves. The serum and colostrum antitoxin levels of the dam were also determined at calving.

No antitoxin was present at birth and before suckling, but at 2 days old it was present in the serum of all the calves and generally in a low titre. While in 14 calves no antitoxin was present in the blood at 7 days, in the remainder it could be detected for a longer period, even up to 8 weeks, and its persistence was directly related to the titre of the dam's blood and colostrum at calving.

*Antitoxin in Milk*

*During lactation.* Composite milk samples were examined at the same time as blood samples from 105 of the cows recorded in Table 1. Only 6 (5·7 per cent) of the samples showed antitoxin at a titre of 0·25 units/ml. or over. A low incidence of antitoxin in milk samples was also shown by Miller and Heishmann (1943) who found that out of 60 composite samples tested, 1 (1·6 per cent) contained 1 unit/ml. or over. Where antitoxin was found in milk samples, there was an accompanying high antitoxin level in the blood of the cow, usually of 16 to 28 units/ml.

*At calving.* The low incidence of antitoxin in composite milk samples taken from cows in full lactation suggested that an examination should be made of the secretion at the time of calving when the mammary gland is known to be more permeable to the passage of antitoxin. The antitoxin content of the mammary secretion and blood of 25 cows was therefore determined at calving and at 3-day intervals after calving.

It was found that $\alpha$ and $\beta$ antitoxins were present in the colostrum in all except two of these on the day of calving and that the titre was the same as that present in the blood but in 4 samples was higher. Antitoxin was found only for 3 to 6 days subsequent to calving in the secretion of normal cows, but in those developing clinical matistis, it persisted for a longer period (Table 2). Thus in 6 clinical cases developing among a total of 77 cows tested at calving antitoxin, either $\alpha$ or $\beta$, was present in high titre at 6 days and in one of these it was found 2 months after calving.

*Relationship between the Presence of $\alpha$ Antitoxin in Quarter Milk Samples and other Factors*

For this part of the investigation a more sensitive test for antitoxin was employed in order to determine the relationship between its presence and (a) staphylococcal infection of the udder, (b)

alkalinity of the sample and (c) the antitoxin titre of the blood. A total of 239 quarter samples from 60 milking cows were tested for the presence of α antitoxin as well as blood samples taken at the same time. Milk samples were examined culturally for pathogenic organisms, especially staphylococci, and for abnormal alkalinity.

TABLE 2

EXAMPLES OF ANTITOXIN TITRE (UNITS/ML.)
Blood and Milk of cows at calving

| | Test | Blood | | Milk | |
|---|---|---|---|---|---|
| | | α | β | α | β |
| *Normal cow* | 20 days before calving | 4 | 4 | — | — |
| | 12 „ „ „ | 2 | 4 | — | — |
| | At calving | 4 | 8 | 4 | 4 |
| | 3 days after calving | 4 | 4 | 1 | 2 |
| | 6 „ „ „ | 8 | 8 | 1 | 1 |
| | 9 „ „ „ | 8 | 8 | <0·25 | <0·25 |
| *Cow developing mastitis* | 20 days before calving | 8 | 8 | — | — |
| | 12 „ „ „ | 8 | 4 | — | — |
| | At calving | 4 | 8 | 4 | 4 |
| | 2 days after calving (mastitis) | | | | |
| | 3 „ „ „ | 32 | 4 | 8 | 1 |
| | 6 „ „ „ | 32 | 1 | 32 | 0·5 |
| | 9 „ „ „ | >32 | 1 | >32 | 0·25 |

*Staphylococcal infection.* Of the 239 quarter milk samples examined, 175 (73 per cent) contained α antitoxin at a titre of 0·025 units/ml. or over. It will be seen in Table 3 that only 4 out of 88 samples from non-infected cows showed an antitoxin titre above 0·1 units/ml. and for this reason, this titre may be regarded as normal. By this criterion 65 (69 per cent) of 95 infected quarters showed a normal antitoxin content and 30 (31 per cent) were abnormal. The 4 samples from non-infected cows were taken at the time of calving from one cow having a very high blood titre. There is a significant difference ($\chi^2 20$, $p = <0{\cdot}001$) in the incidence of abnormal antitoxin titres between quarters of infected and non-infected cows. In the case of infected quarters, however, the incidence is much lower than that found by Lasmanis and Spencer (1954).

*Alkalinity of the milk.* In Table 4, it will be seen that of 73 samples

with abnormal alkalinity 23 (31 per cent) showed titres of 0·2 units/ml. or over, while of 166 samples with a normal reaction, 19 (11 per cent) also had high titres ($\chi^2$12·7, p=<0·001). Of 23 samples having abnormal antitoxin and alkalinity, 20 were infected with staphylococci, while disturbance of the udder was caused by *Str. dysgalactiae* in 3. Thus the presence of high antitoxin in quarters with abnormal alkalinity may be due to causes other than the presence of specific infection due to staphylococci.

TABLE 3

RELATIONSHIP BETWEEN ANTITOXIN TITRE OF THE MILK AND INFECTION

| | *Quarters* | *α antitoxin (units/ml.) titres present in milk* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *<0·025* | *0·025* | *0·05* | *0·1* | *0·2* | *0·5* | *1·0* | *2·0* | *>2·0* |
| Infected cows (38) | +95 | 22 | 5 | 21 | 17 | 11 | 12 | 2 | 4 | 1 |
| | −56 | 13 | 5 | 17 | 13 | 2 | 4 | 1 | 0 | 1 |
| Non-infected cows (22) | −88 | 29 | 13 | 17 | 25 | 0 | 0 | 4 | 0 | 0 |
| Total (60) | 239 | 64 | 23 | 55 | 55 | 13 | 16 | 7 | 4 | 2 |

TABLE 4

RELATIONSHIP BETWEEN ANTITOXIN TITRE OF THE MILK AND ALKALINITY

| *Alkalinity* | *Quarters* | *Antitoxin titre of milk (units/ml.)* | |
|---|---|---|---|
| | | *0·1 or less* | *0·2 or more* |
| Abnormal .. .. .. | 73 | 50 | 23 (31%) |
| Normal .. .. .. | 166 | 147 | 19 (11%) |

TABLE 5

RELATIONSHIP BETWEEN ANTITOXIN TITRE OF THE MILK AND THE BLOOD

| *Blood titre* | *Cows* | *Quarters* | *Antitoxin titre of milk (units/ml.)* | |
|---|---|---|---|---|
| | | | *0·1 or less* | *0·2 or more* |
| 1–4 units/ml. | 46 | 183 | 177 | 6 (3·2%) |
| 8–32 units/ml. | 14 | 56 | 20 | 36 (64%) |

*Antitoxin titre of the blood.* Blood samples from the 60 cows were taken at the same time as quarter milk samples and tested for antitoxin content. In Table 5 the cows have been arbitrarily divided into two groups according to their blood antitoxin titres. It will be seen that a much higher proportion of quarters with high milk titres belonged to cows with accompanying high blood titres than to those possessing low blood titres.

## DISCUSSION

The results of this study show that the presence of staphylococcal antitoxin in the blood is stimulated by the presence of a pathogenic species of staphylococcus. They also confirm the view expressed by Minett (1937) that in the cow one of the sites providing a stimulus to antitoxin production is the infected udder. The evidence in support of this is derived from a number of observations. Firstly there is the finding that the antitoxin titre of the blood increases with age and that the rate of infection of the udder with staphylococci in succeeding lactations also increases. Secondly, evidence of the specific nature of the response was provided by finding that the type of antitoxin in the blood corresponded to the toxigenic character of the staphylococci prevailing in the udder. Thirdly, a marked stimulus to antitoxin production occurred only in cows developing mastitis due to staphylococci. There must, however, be sites of infection other than the udder since a small number of cattle showed antitoxin in the blood before commencing their first lactation. Evidence to this effect in another species was provided by cultural examinations of various organs of 48 pigs at slaughter. Staphylococci, predominantly of $\beta$ type were found in the lungs and respiratory tract and gave rise to $\beta$ antitoxin in the blood.

Antitoxin is present usually in low concentration in the milk of normal udders, but its titre is increased in the colostrum at calving when the epithelium of the mammary gland becomes more permeable to the passage of antibodies from the blood. It persists at the level present in the blood for only a few days in the normal cow, but in quarters developing mastitis due to staphylococci it may be present for up to 2 months. The presence of antitoxin in milk is not, however, specifically associated with such infection since, during lactation, antitoxin levels above normal may sometimes be found where there is abnormality resulting from other infections, e.g. *Str. dysgalactiae*.

This investigation shows that antitoxin in milk is not in itself an index of protection against infection of the udder with staphylococci since it was present more frequently in infected quarters than in those free from infection. It was also present in high titre in the blood and milk of cows developing clinical mastitis. This does not necessarily mean that the presence of antitoxin is of no immunological value to the cow since it may prevent the onset of a toxaemia

which might arise as a result of infection with a toxin-producing staphylococcus such as the prevailing 42D phage type.

## CONCLUSIONS

Staphylococcal antitoxin was present in the blood of 159 (79 per cent) of 202 cattle and its titre increased with the age of the animal.

Antitoxin response in the blood was specifically related to the toxigenic character of the staphylococcus present in the udder of the cow or in the respiratory tract of the pig.

The blood titre did not increase at the time of calving except in cows developing clinical mastitis due to staphylococci.

Antitoxin was present in very low concentrations in the milk of cows in full lactation, but in the colostrum its titre was equal to that of the blood. It remained at this titre for a few days only in normal cows, but in those developing staphylococcal mastitis it persisted for a longer period.

No definite correlation between increased antitoxin titre of the milk and the presence of staphylococcal infection was found, but there was a close relationship with a high blood titre.

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[*Received for publication, August 16th,* 1958]

# OBSERVATIONS ON THE EFFECTS OF GONADECTOMY AND SUBSEQUENT INJECTION OF TESTOSTERONE ON THE ADRENALS OF MALE SHEEP

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## INTRODUCTION

The existence of a functional relationship between corticoadrenal tissue and the manifold processes of reproduction has been widely acknowledged since Howard (1927) and Deanesly (1928) showed that the so-called X zone of the mouse adrenal undergoes distinctive degenerative changes which are associated with the onset of puberty in the male and first gestation in the female. Many workers have investigated this hypothesis of a link-up between the adrenals and the gonads. Korenchevsky and Hall (1938), for instance, demonstrated that recognisable changes occur in the intracellular lipoid pattern of the rodent adrenal which can be linked with alterations in sexual activity. The literature on adrenal-gonad relationships is vast, but comprehensive summaries of the problem can be found in the works of Parkes (1945), Burrows (1949) and Courrier, Baclesse and Marois (1953).

Whilst both gonad and adrenal perform distinct physiological roles, the two glands possess a common denominator in that the adrenal cortex depends on pituitary adrenotrophin for normal functioning and gonadal tissue on pituitary gonadotrophins. Whether or not a further interdependence exists between the glands by virtue of their common coelomic epithelial origin is debatable, but variations in staining reaction which are attributable to sexual dimorphism have been recorded in the rat by Nelson (1941) and also in normal, castrated and spayed cats by Lobban (1952). The latter worker pointed out, however, that is not possible to correlate structural variations in the adrenal with a simple sexual dimorphism, for alterations in staining affinity and indeed, cortical morphology as a whole, turn primarily on the innermost zone of the cortex, the zona reticularis.

Zuckerman (1941, 1953), Parkes (1945) and Mandl (1951) have established that in the female animal there is a close relationship between corticoadrenal tissue and the various phases of the oestrous and menstrual cycles. Jones (1957) in his symposial work on the adrenal cortex states that gonadectomy results in an alteration of size of the adrenal cortex, the zona reticularis becoming thicker in the castrated male and the same zone declining in the spayed female. It could be surmised from this that, as Lobban suggests, the zona reticularis functions as some kind of secondary sex organ and so reflects changes in the sexual condition of the animal in question as, for example, do the epithelium of the uterus and the seminal vesicles. The fact that injection of gonadal hormones reverses these changes as recorded both by Lobban and Jones, lends considerable weight to this hypothesis.

It was decided to investigate whether the experimental alteration

of sex and also the subsequent injection of testosterone influenced in any way the zona reticularis of the adrenal gland of sheep.

## METHODS AND MATERIAL

Four Soay sheep of approximately six months of age were castrated. On recovery from the operation three of the animals were given intramuscular injections of testosterone propionate. The remaining sheep was used as a control. The three experimental animals were given 200 mg., 400 mg., and 600 mg. of testosterone respectively (Table 1). Each of the experimental animals was destroyed 72 hours after its last injection of testosterone. The control animal was killed at the same time as No. 3.

TABLE 1

| *Days* | *Sheep 1* | *Sheep 2* | *Sheep 3* | *Sheep 4 (control)* |
|---|---|---|---|---|
| 1 | X | X | X | — |
| 2 | — | X | X | — |
| 3 | — | — | X | — |
| 4 | Death | — | — | — |
| 5 | | Death | — | — |
| 6 | | | Death | Death |

X represents a single dose of 200 mg. testosterone propionate

Both adrenals were removed from each animal immediately after death and fixed in Baker's formol-calcium (Baker, 1946). Paraffin sections were cut at 7 μ and taken longitudinally through the middle of each gland. Since the zona reticularis of the sheep adrenal normally displays a noticeable acidophilia, Azan stain was used throughout in order to facilitate definition of the region.

The relative proportions of the different zones of the cortex of the four experimental animals and also six normal adult uncastrated male subjects were determined at their thickest and thinnest portions and thus the average width of the individual zones deduced. In order to give as accurate an overall picture as possible, in the experimental glands the average zonal widths were deduced from a series of six sections cut from the mid-portions of each gland and compared with the readings obtained from the glands of the six uncastrated animals. The most convenient method of calculating the zonal measurements was to project the whole slide on to a screen and to measure the actual zones with a foot-rule. Thus, in the tables the width of the zones in the different glands is expressed as the number of divisions of the foot-rule which they were seen to occupy under standard conditions of projection and magnification. From this the ratio of the width of the zona fasciculata to that of the zona reticularis was deduced for each gland, and the mean fasciculata/reticularis (F/R) ratio used as a gauge for comparison of the variation in the latter zone met with in the five different classes of animals used for this study.

## RESULTS

With the Azan stain, the junction between fascicular and reticularis tissue is generally quite distinct, the cells of the latter zone being acidophilic while those of the former possess a much paler staining cytoplasm. In all of the slides examined the division between fasciculata and reticularis was taken as the junction between the pale staining cells and those with acidophilic cytoplasm.

TABLE 2

CLASS 1—ADULT, NON-CASTRATED MALES

| | *Z.G.* | *Z.F.* | *Z.R.* | *F/R ratio* |
|---|---|---|---|---|
| Sheep A .. | 10 | 55 | 26 | 2·12/1 |
| Sheep B .. | 11 | 66 | 28 | 2·36/1 |
| Sheep C .. | 18 | 61 | 25 | 2·44/1 |
| Sheep D .. | 9 | 67 | 23 | 2·91/1 |
| Sheep E .. | 9 | 71 | 23 | 3·09/1 |
| Sheep F .. | 10 | 69 | 32 | 2·16/1 |

Mean F/R ratio for Class 1 Sheep—2·51/1

In the Tables the figures represent divisions of a foot-rule at standard magnification. *Z.G.*—Zona Glomerulosa. *Z.F.*—Zona Fasciculata. *Z.R.*—Zona Reticularis. *F/R ratio*—Fasciculata/Reticularis ratio.

TABLE 3

CLASS 2—SHEEP G, ADULT CASTRATED CONTROL

| | *Z.G.* | *Z.F.* | *Z.R.* | *F/R ratio* |
|---|---|---|---|---|
| Section 1 .. | 12 | 49 | 45 | 1·09/1 |
| Section 2 .. | 12 | 41 | 39 | 1·05/1 |
| Section 3 .. | 11 | 46 | 45 | 1·02/1 |
| Section 4 .. | 12 | 47 | 46 | 1·02/1 |
| Section 5 .. | 12 | 46 | 45 | 1·02/1 |
| Section 6 .. | 12 | 46 | 44 | 1·05/1 |

Mean F/R ratio for Class 2 Sheep—1·04/1

The sheep on which observations were based were divided into the five classes according to their sexual condition: Class 1, normal uncastrated males (sheep A to F): Class 2, adult castrated control (sheep G): Class 3, adult castrate with previous administration of 200 mg. testosterone propionate (sheep H): Class 4, adult castrate with previous administration of 400 mg. testosterone propionate

(sheep I): Class 5, adult castrate with previous administration of 600 mg. testosterone propionate (sheep J). In all classes of sheep, the width of the zona glomerulosa showed very little variation and as the small amount of variation in width in the zona fasciculata in the different animals was due largely to differences in actual gland size, this factor was adjudged to be normal and was accordingly disregarded.

TABLE 4

CLASS 3—SHEEP H, ADULT CASTRATE INJECTED WITH 200 MG. TESTOSTERONE

| | *Z.G.* | *Z.F.* | *Z.R.* | *F/R ratio* |
|---|---|---|---|---|
| Section 1 .. | 8 | 24 | 11 | 2·18/1 |
| Section 2 .. | 7 | 23 | 12 | 1·92/1 |
| Section 3 .. | 7 | 23 | 11 | 2·09/1 |
| Section 4 .. | 7 | 26 | 13 | 2·00/1 |
| Section 5 .. | 7 | 23 | 10 | 2·30/1 |
| Section 6 .. | 8 | 24 | 12 | 2·00/1 |

Mean F/R ratio for Class 3 Sheep—2·08/1

TABLE 5

CLASS 4—SHEEP I, ADULT CASTRATE INJECTED WITH 400 MG. TESTOSTERONE

| | *Z.G.* | *Z.F.* | *Z.R.* | *F/R ratio* |
|---|---|---|---|---|
| Section 1 .. | 8 | 43 | 20 | 2·15/1 |
| Section 2 .. | 9 | 46 | 23 | 2·00/1 |
| Section 3 .. | 10 | 46 | 23 | 2·00/1 |
| Section 4 .. | 9 | 46 | 21 | 2·19/1 |
| Section 5 .. | 9 | 48 | 23 | 2·09/1 |
| Section 6 .. | 9 | 47 | 21 | 2·24/1 |

Mean F/R ratio for Class 4 Sheep—2·11/1

The width of the zona reticularis, however, showed marked variation in the individual classes despite the fact that the F/R ratio was the same for right and left glands in any one animal. It can be seen from the Tables that this ratio, i.e., the width of the fasciculata to that of the reticularis presents certain characteristics which can be correlated with the experimental condition of the different classes of animals.

TABLE 6

CLASS 5—SHEEP J, ADULT CASTRATE INJECTED WITH 600 MG. TESTOSTERONE

| | *Z.G.* | *Z.F.* | *Z.R.* | *F/R ratio* |
|---|---|---|---|---|
| Section 1 .. | 6 | 333 | 16 | 2·06/1 |
| Section 2 .. | 6 | 31 | 14 | 2·21/1 |
| Section 3 .. | 6 | 29 | 14 | 2·07/1 |
| Section 4 .. | 6 | 333 | 15 | 2·20/1 |
| Section 5 .. | 7 | 29 | 15 | 1·93/1 |
| Section 6 .. | 6 | 34 | 15 | 2·27/1 |

Mean F/R ratio for Class 5 Sheep—2·12/1

TABLE 7

TABULATION OF MEAN F/R RATIOS

| *Class* | *State of animal* | *F/R ratio* |
|---|---|---|
| 1 | Non-castrated adult male | 2·51/1 |
| 2 | Castrated adult male control | 1·04/1 |
| 3 | Castrate, 200 mg. testosterone | 2·08/1 |
| 4 | Castrate, 400 mg. testosterone | 2·11/1 |
| 5 | Castrate, 600 mg. testosterone | 2·12/1 |

## DISCUSSION

From the measurements recorded in the Tables, it can be said that a zona reticularis is present in the adrenal cortex of male sheep whether castrated or not, and that the zone undergoes structural alterations which, when put into ratio with a relatively constant factor such as the width of the zona fasciculata, can be seen to bear a direct relationship to the sexual condition of the animal in question. That the zona reticularis can be directly correlated with gonadal activity in sheep is in agreement with the findings of Nelson (1941) in the rat, Bennet (1940) and Lobban (1952) in the cat, and also what is said by Jones (1957) to be the general case in that group of animals, of which the sheep is a member, which normally display post-pubertal, adrenal sexual dimorphism.

The F/R ratio of the adrenal cortex of normal adult uncastrated male sheep is 2.51/1: in other words there is approximately two and a half times more fascicular tissue than there is reticular. The changes which can be observed in the zona reticularis of castrated animals, manifested by fluctuations in their F/R ratios, can be sum-

marized. In the adult castrated animal the zona reticularis shows a very marked degree of development, so much so that calculation of the F/R ratio indicates that gonadectomy in male sheep induces expansion of the zone to approximately the same width as that of the fasciculata.

Injection of testosterone in castrate sheep brings about a decrease in width of the reticularis and it is apparent from the F/R ratios calculated for the injected castrate animals that this decrease in width caused by the administration of the hormone is progressive. Thus the F/R ratio for the animal which received 200 mg. of testosterone shows that the reticularis is approximately half the width of the fasciculata. The F/R ratios of the 400 mg. and 600 mg. animals indicate that the reticularis has decreased even more in width and, taking this progressive reduction in zonal width a step further, it is quite reasonable to suppose that if the scope of the experiments had been widened, doses of testosterone higher than 600 mg. might well have yielded an F/R ratio of approximately $2\frac{1}{2}/1$ which was the F/R ratio obtained for normal uncastrated male sheep.

## CONCLUSIONS

An investigation on the effects of gonadectomy and subsequent administration of testosterone propionate on the adrenal glands of adult male sheep is reported.

The adrenal cortex of male sheep whether castrated or not possesses a definitive zona reticularis.

Structural alterations in the width of the zona reticularis have been demonstrated which can be correlated with the sexual condition of the sheep under experimental conditions.

The ratio of the width of the zona fasciculata to that of the zona reticularis in five different classes of animals is as follows: 2·51/1 in non-castrated adult males; 1·04/1 in castrated adult males; 2·08/1 in castrate males injected with 200 mg. testosterone; 2·11/1 in castrate males injected with 400 mg. testosterone; 2·12/1 in castrate males injected with 600 mg. testosterone.

## ACKNOWLEDGMENTS

The writer's thanks are due to Mr. T. Grahame, T.D., F.R.C.V.S., F.R.S.E., Head of the Department of Veterinary Anatomy, for his supervision and very helpful advice in the preparation of this paper.

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[*Received for publication, September 5th,* 1958]

# FAMILIAL CEREBELLAR DEGENERATION AND ATROPHY—A SEX-LINKED DISEASE AFFECTING LIGHT SUSSEX PULLETS

By

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## INTRODUCTION

Hereditary degenerations of the cerebellum occur in many species of livestock (Saunders, 1952). In chickens similar diseases have been reported by Scott, Morrill, Alberts and Roberts (1950) and Winterfield (1953). The present paper records the pathology and genetics of a cerebellar degeneration recently encountered in Light Sussex pullets.

## RESULTS

### *Field History*

The disease first appeared in the progeny of a breeding flock. The owner reported that, when he mated Light Sussex pullets with Brown Leghorn cockerels, the chicks were normal; but that, when he mated the same females with Light Sussex cockerels, some of the offspring developed mild ataxia and tremors of the head and neck when between one and two months of age. Only pullets were affected. When some of the same breeding pullets were mated with Light Sussex cockerels from another source trembling pullets were still obtained. The disease did not appear to be contagious as chickens of other breeds reared alongside those affected remained normal. Twelve customers supplied by this breeder with day-old chicks from the pure Light Sussex mating complained of the disease, reporting that only pullets were affected and giving the incidence as from five to ten per cent. There was no expression of dissatisfaction with the birds apart from the nervous symptoms; they were said to rear normally and to lay satisfactorily. Post-mortem examination of several birds revealed a diminutive cerebellum but no other evidence of disease.

### *Breeding Experiment*

As the field history suggested the likelihood of a genetic basis for the disease, a breeding experiment was carried out. Five affected pullets from the original flock were mated with a Rhode Island Red, laboratory cockerel. Egg production was only moderate and fertility low—during a period of twenty weeks only 103 eggs were laid of which only 26 were fertile. Seven of these hatched to

produce four pullets and three cockerels, of which three pullets and two cockerels survived to maturity without showing any symptoms of the disease.

These two cockerels were then mated with the pullets and also with the original affected hens. The latter mating failed to produce a single fertile egg. The results of the pullet matings are shown in Table 1. Again the matings were relatively infertile. The disease was recognised in eleven pullets, but the ten cockerels reared were all normal.

TABLE 1

MATINGS OF OFFSPRING OF AFFECTED FEMALES

| *Pullets* | *Cockerels* | *Eggs* | *Fertile* | *Hatched* | | | *Survived over 3 months* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | ♂ | | ♀ | |
| | | | | ♂ | ♀ | *Total* | *Normal* | *Affected* | *Normal* | *Affected* |
| 1 | A | 92 | 65 | 14 | 21 | 35 | 6 | 0 | 6 | 6 |
| 2 | A | 18 | 15 | 4 | 4 | 8 | 3 | 0 | 0 | 4 |
| 3 | B | 12 | 8 | 2 | 1 | 3 | 1 | 0 | 0 | 1 |
| | Total | 122 | 88 | 20 | 26 | 46 | 10 | 0 | 6 | 11 |

In the third stage of the experiment, the same two cockerels were mated with four of these affected pullets and with another (No. 8 in Table 2) which had been subsequently obtained from the original breeder. The results are shown in Table 2. As in the first attempt to breed from affected females, fertility was very low, but the disease reappeared and, as before, only in pullets.

The two main conclusions derived from the mating tests are that the disease was transmitted by breeding and that it occurred only in females. The small number of birds reared to the age at which the disease could be recognised renders further interpretation difficult, but a sex-linked recessive appears to fit the facts. Affected hens would then each carry the Y chromosome and an X chromosome containing the mutant gene. Pullets 1, 2 and 3 would be normal, but the cockerels A and B would be carriers. When they were intermated half the females produced would, therefore, be affected and half normal. Since the males would each receive a non-mutant containing X chromosome from their dams, none would show the disease, although half would be carriers. Actually seventeen females were reared of which eleven were affected. Ten males were reared, none of which was affected with the disease.

In the last part of the experiment, cockerels A and B were mated to the affected pullets. Again one would expect half the

females to be affected. As for the males, each would receive a mutant-bearing X chromosome from its dam, while half would also receive a similar chromosome from the sire, so that half the males would have the disease. Of the nine females raised, six were affected. No affected males were produced, possibly due to insufficient numbers or to the gene being lethal in double dose. The reasons for both the low fertility and hatchability were not established.

TABLE 2

MATINGS OF SONS OF AFFECTED HENS TO AFFECTED PULLETS

| *Pullets* | *Cockerels* | *Eggs* | *Fertile* | *Hatched* | *Survived over 3 months* | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ♂ | | ♀ | |
| | | | | | *Normal* | *Affected* | *Normal* | *Affected* |
| 4 | A | 43 | 14 | 8 | 1 | 0 | 1 | 3 |
| 5 | A | 25 | 11 | 5 | 2 | 0 | 0 | 1 |
| 6 | B | 15 | 5 | 2 | 0 | 0 | 1 | 0 |
| 7 | B | 6 | 1 | 0 | 0 | 0 | 0 | 0 |
| 8 | B | 25 | 12 | 6 | 3 | 0 | 1 | 2 |
| Total | | 114 | 43 | 21 | 6 | 0 | 3 | 6 |

*Clinical Observations*

Symptoms usually first appeared when the chickens were between two and three months of age. Occasional rippling tremors of the head and neck were observed when the pullets were at rest. When disturbed they became moderately ataxic, placing their feet wide apart in order to remain upright, while the tremors became more persistent and more marked. A month later the tremors were present frequently and intermittently and were readily seen. The nervous symptoms did not appear to cause any serious inconvenience or to interfere with normal growth and rearing. In pullets kept for breeding the symptoms persisted unchanged into adult life. One of the original cases survived over four years still showing the tremors of the head and neck and the slightly straddling gait that compensated for the reduced ability to maintain balance.

*Morbid Anatomy*

The material studied comprised the original field cases and all the birds produced in the breeding experiment. In all, 26 affected birds from three months to over two years of age were examined.

No pathological changes of the nervous system were found in any of the clinically unaffected birds from the breeding experiment. Ten per cent formol-saline was used routinely as the fixative at first, but was later replaced by formol-ammonium bromide. Zenker's fixative was occasionally employed, when it was intended to stain sections by Mallory's phosphotungstic acid haematoxylin. Paraffin sections were prepared in the usual way. Ehrlich's haematoxylin followed by eosin was the routine staining method, special neurological methods being applied as required.

Gross abnormality was confined to the cerebellum, the variation in size ranging from about a half normal (Fig. 1) to a tiny shrunken nodule of tissue (Fig. 2). Even the most diminutive cerebellum retained its distinctive shape and gross structure; folia and sulci were perfectly recognisable in both the intact and sliced organ, but the folia were much reduced in size.

Microscopical changes are confined to the cerebellum and, while differing in points of detail, both between cases and in the same specimen, are essentially the same in all cases studied. The folia are thinner than normal, extremely so in advanced cases (Fig. 3), when all the anatomical layers appear to be reduced, especially the molecular layer. In one case uneven shrinkage has led to separation of contiguous folia in the depths of the sulci. The central core of myelinated fibres is almost entirely normal. Although in advanced cases there appears to be some reduction in its thickness, the fibres themselves (as demonstrated by Romanes' technique) and their myelin sheaths (Loyez's haematoxylin, copper phthallocyanin) are not demonstrably abnormal either here or in the region of the central nuclei. Occasionally swollen, degenerating fibres can be seen in the cerebellar peduncle. Sometimes a transected venule gapes where it penetrates the white matter (Fig. 3). In a few sections isolated venules bear an irregular cuff of lymphocytes, while some show a slight hyperchromasia or proliferation of the endothelium —the only signs of any inflammatory response. The granular layers appear to be normal in several cases. Where abnormality can be seen it takes the form of decrease in the number of granule cells with consequent rarefaction of the layer and variable decrease in its thickness. A few shrunken granule cells are visible, but not as many as one would expect to find in a rapidly active lesion.

By far the major abnormality affects the Purkinje cells and the arborisation of the dendrons which forms the basis of the molecular layer. In the worst affected sections of cerebellum very few Purkinje cells have survived and those that have are shrunken and degenerate. In only moderately affected sections many normal Purkinje cells are seen, with variable gaps where others have been. Various degenerate forms are visible, ranging from local shrinkage of the normally granular cytoplasm from the cell wall to a completely clear space surrounding nucleus and cytoplasm and hyperchromatic shrinking

of the entire cell body. Silver impregnation by Romanes' method reveals that between the extremes of healthy dendrons issuing from healthy Purkinje cells (Fig. 4) and the complete absence of Purkinje cells and dendrons (Fig. 5), there exists a variety of swollen dendrons issuing from still visible cells and swollen fragments detached from the parent cells (Figs. 5 and 6). With the increasing destruction of Purkinje cells there appears to be an increase in the density of basket fibres so that, in some sections, the Purkinje cell layer is replaced by a prominent palisade of thickened basket fibres (Fig. 7). In advanced cases they frequently fragment and all but disappear (Fig. 8).

The molecular layer varies from its normal structure and thickness to being a virtually structureless thin rind on the folia. The greater the degree of Purkinje cell destruction, the thinner the rind (Fig. 5). The more advanced the disease, the less distinct is the boundary between the granular and molecular layers in the haematoxylin and eosin stained sections. Several factors appear to be responsible for this: the absence of the normal layer of Purkinje cells, the depletion of the usually numerous granule cells, the apparent proliferation of Bergmann cells and a variable apparent increase in the concentration of cell nuclei in the molecular layer. Gold impregnation of suitably fixed material has demonstrated astrocytes normally disposed throughout the granular layer and white matter, but only occasionally present in the molecular layer. No gliosis of any kind, or collagen or reticulin scarring could be demonstrated in the molecular layer, although vertical fibres are visible occasionally in the gold preparations apparently emanating from granule cells. So far as can be seen the molecular layer consists of fibres from basket cells and occasionally granule cells, sparse argentiphilic tangential fibres which are usually well preserved (Figs. 6 and 7), degenerating Purkinje cell dendrons and, where these have disappeared, a grey felted background which takes the silver very weakly. Against this background can be seen scattered small nerve cells in varying number and occasional astrocytes and microgliacytes. The occasional increased concentration of nuclei here is interpreted as due to collapse of the normal matrix. The leptomeninges are not visibly diseased, but they tend to detach locally from the free, ragged surface of degenerate parts of the folia. No abnormalities have been seen in the sections of medulla oblongata.

A remarkable feature of the disease we have described is the almost complete absence of mesodermal reaction. This would appear to suggest that the disease is a true hypoplasia, but it is the only evidence supporting such a theory. The apparent normality of the chicks on hatching and the insidious development of symptoms imply a slow decline of cerebellar function accompanied by the gradual development of cerebral compensation without which it would be impossible for affected adults to lead a nearly normal existence. Histologically the disease expresses itself as a slow degeneration of Purkinje cells with consequent swelling and dis-

integration of their dendrons leading to collapse of the molecular layer and disappearance of their axons leading to a decrease in the white matter. The changes in the granular layer may be secondary to this. No explanation is offered for the absence of phagocytosis of the degenerating and dead tissue elements and their replacement by scar tissue, but their degeneration is plainly visible. For this reason, although the disease was initially diagnosed as a hypoplasia, it is now presented as familial cerebellar degeneration and atrophy.

## DISCUSSION

The disease appears to be comparable if not identical to that described by Winterfield (1953) in the New Hampshire meat strain, which occurred in pullets of about 12 weeks of age in fifteen separate flocks. Symptoms were bobbing and weaving and fine tremors of the head and neck. Some birds became ataxic if excited, but severe inco-ordination was not observed. The incidence varied from three to ten per cent; mortality was nil. Several pullets approximately seven months old were examined and found to be in good bodily condition, three of them being in lay. In six of these pullets a diminutive cerebellum was found at autopsy, about an eighth to a quarter the normal size for that age and about half the size of the normal cerebellum of a day-old chick. The folia were indistinct and the sulci extremely shallow. Microanatomical changes were confined to the cerebellar cortex: the molecular and granular layers were exceedingly thin, many Purkinje cells were degenerate while, in some sections, many had disappeared. The onset of symptoms at three months was interpreted as evidence of atrophy in addition to hypoplasia. From the evidence available Winterfield thought that a sex-linked recessive factor was the possible cause.

The disease also has some points of similarity to the "shaker fowl" as described by Scott *et al.* (1950), but is less severe. In their cases the time of onset was earlier, at four weeks. By fourteen weeks, most of the birds could no longer stand and died of inanition. Only a few were reared to maturity. No mention was made of gross cerebellar degeneration, but microscopically Purkinje cell degeneration and destruction were present. The data suggested the disease was inherited as a sex-linked recessive. Kawahara (1955) reported a disease in Barred Plymouth Rocks, which he considered similar to the "shaker" syndrome.

Comparable diseases occur in man (Greenfield, 1954) and other animals (Saunders, 1952), of which Holmes' familial cerebello-olivary degeneration and the cortico-cerebellar atrophies of sheep (van Bogeart and Innes, 1950; Innes, Rowlands and Parry, 1949) and of cattle (Innes, Russell and Wilsdon, 1940) are most closely related morphologically. The possibility of a hereditary basis has long been realised.

## CONCLUSIONS

A disease is described which occurred in young Light Sussex pullets. Clinically it appeared as slight ataxia with fine tremors of the head and neck. Histologically the symptoms were referable to degeneration of the cerebellar Purkinje cells and nerve cells of the granular layer, with consequent atrophy of the cerebellum. The disease was transmitted by breeding and appeared to be associated with a sex-linked recessive gene.

## ACKNOWLEDGMENTS

The histological preparations were made by technical staff of the Pathology Department at the Central Veterinary Laboratory, under the direction of N. C. Lacey, F.I.M.L.T. The photograph for Fig. 1 was taken by J. T. Done, B.V.Sc., M.R.C.V.S., those for Figs. 7 and 8 by E. C. Clements, A.I.B.P.

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[*Received for publication, September 2nd,* 1958]

## LEGENDS TO ILLUSTRATIONS

Fig. 1. The cerebellum of the brain on the left is about half the normal size. The normal brain on the right is from a slightly older bird.

Fig. 2. Extreme atrophy of the cerebellum on the right.

Fig. 3. Atrophied cerebellar folia. × 57.

Fig. 4. Normal Purkinje cells. × 470.

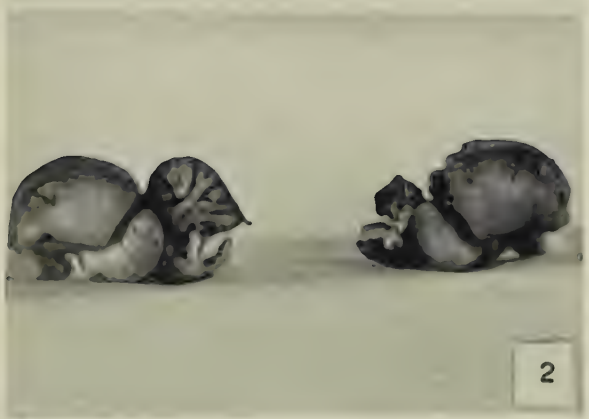
Fig. 5. On the right the Purkinje cells have disappeared, their former position being now occupied by proliferated basket fibres. On the left Purkinje cell degeneration is still active, the swollen, fragmenting dendrons being visible. Observe the difference in thickness of the molecular layer in the two zones. × 100.

Fig. 6. On the left swollen fragments of Purkinje cell dendrons. On the right a Purkinje cell with its main dendron trunk markedly swollen. A few tangential fibres are well preserved. × 470.

Fig. 7. Purkinje cells have disappeared, their site being marked by well developed, empty baskets. Tangential fibres are well preserved. × 280.

Fig. 8. Purkinje cells have disappeared. Basket fibres are sparse, swollen and beginning to disintegrate. × 470.

Fig. 3 is taken from a haematoxylin and eosin stained section. Figs. 4, 5, 6, 7 and 8 are from sections impregnated by Romanes' silver method.

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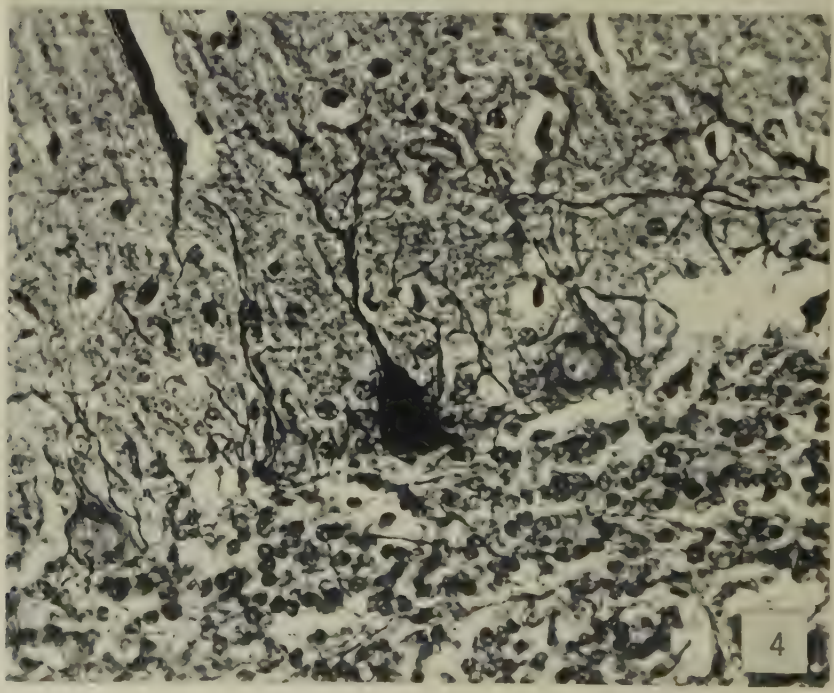
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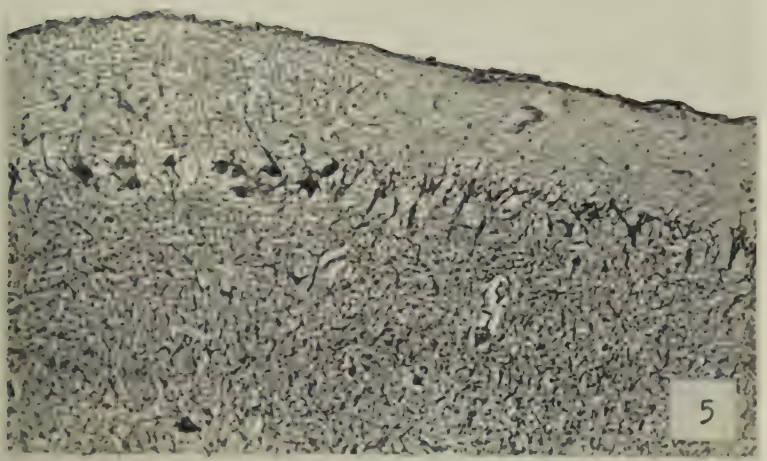
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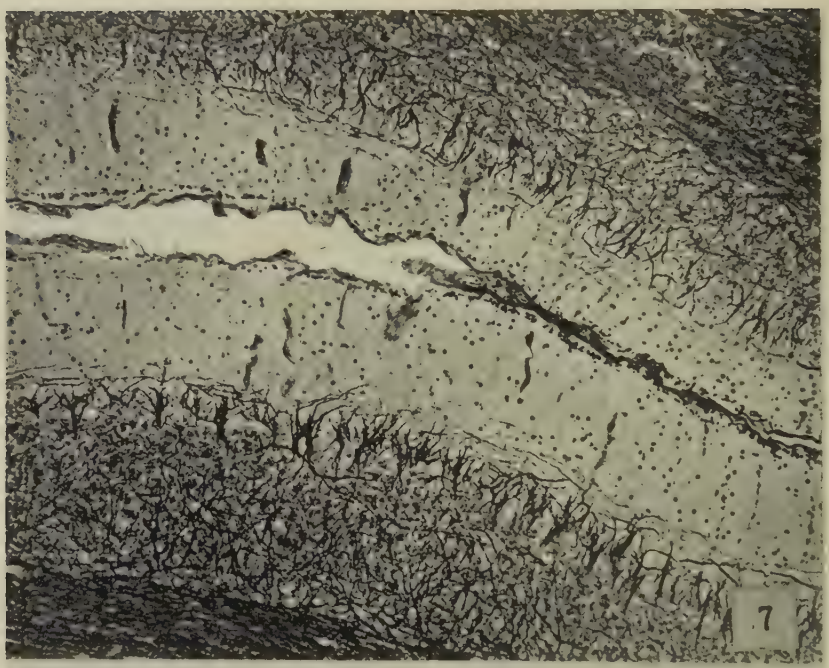
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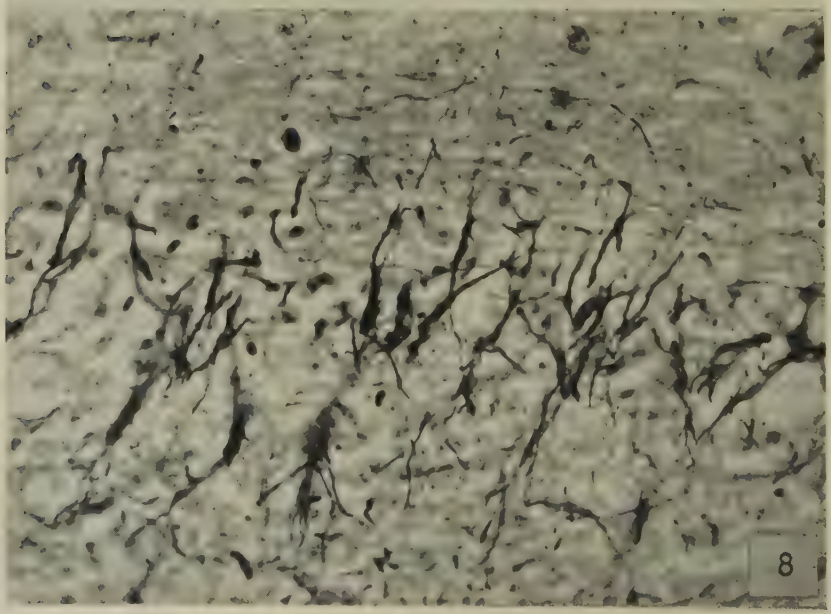
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# STUDIES ON *PASTEURELLA SEPTICA*

## III. Strains from Human Beings

By

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### INTRODUCTION

*Pasteurella septica*, though widely present in the upper respiratory tract of domestic mammals and birds, is generally believed to occur rarely if at all in the healthy human subject. This paper records its isolation from two veterinary students and describes the properties of the strains obtained, together with those of 14 cultures from cases of human pasteurellosis.

### MATERIALS AND METHODS

*Examination of swabs for* Past. septica. Nose and throat swabs were obtained from 71 fourth year students, including four female students, of the Royal Veterinary College, London. The swabs were plated on to 5 per cent ox blood agar and the cultures examined after 24 hours incubation. Each swab was also incubated 24 hours in 5 ml. beef infusion broth, of which 0·5 ml. was then injected subcutaneously into a 20 to 25 g. mouse. The heart blood of each mouse dying within 14 days was cultured on to blood agar.

*Cultures from human pasteurellosis.* Fourteen cultures were obtained from various hospital laboratories and other sources and were freeze-dried until required. The figures in brackets are the strain number. Renal abscess (84), dog bite (136), suppurative pericarditis (149), empyema (N.C.T.C. 8080) (170), dog bite (201), dog bite (202), cat scratch (207), cat bite (612), dog bite (679), cat bite (696), submandibular abscess (709), sputum (710), dog bite (711), sputum (712).

*Examination of strains.* Strains were examined for capsule, mucoid colony formation, iridescence, hyaluronic acid production, acid agglutinability, mouse pathogenicity and carbohydrate fermentation by the methods given in a previous paper (Smith, 1958).

### RESULTS

#### *Examination of Nose and Throat Swabs*

*Past. septica* was isolated from the throat of two of the 71 students examined; both were males. One (39) was positive both to culture and mouse inoculation: a heavy growth of the organism occurred in primary cultures from the swab, and the mouse died 18 hours after inoculation. This student remained a carrier for four months after which no further swabs were taken. The other (33) was positive for a few days by mouse inoculation only, the mice dying 9 to 12 days after inoculation. In no case was *Past. septica* isolated from the nose (see Table 1). Many mice died from infections due to other bacteria,

of which *Streptococcus pneumoniae* (14 isolations) and coliform bacteria (12 isolations) were the commonest.

Both of the students who gave positive results were in good health with no history of serious respiratory disease, though one of them (39) stated that he suffered from frequent colds and had had tonsillitis six months previously. Neither lived in a household where pet animals or birds were kept, but in the course of their studies both frequently handled post-mortem material from large and small animals and came into contact with cases of animal disease.

TABLE 1

ISOLATION OF *PAST. SEPTICA* FROM NOSE AND THROAT SWABS

| *Student number* | *Swab* | *Date of swabbing* | | | |
|---|---|---|---|---|---|
| | | *22.2.55* | *1.3.55* | *16.3.55* | *15.6.55* |
| 33 | Nose | — | — | — | — |
| | Throat | +m | +m | — | — |
| 39 | Nose | — | — | — | — |
| | Throat | +cm | +cm | +cm | +cm |
| Remainder (1-32, 34-38, 40-71) | Nose | — | Not examined | | |
| | Throat | — | | | |

+m indicates positive by mouse inoculation only.
+cm indicates positive by culture and mouse inoculation.

*Properties of Strains*

The results of tests carried out on the 14 cultures from cases of human pasteurellosis and on cultures 275 and 276 (from students 39 and 33 respectively) are given in Table 2. As might have been expected, the strains from dog bites exhibited on the whole the properties previously shown to be characteristic of healthy dog strains (Smith, 1958), i.e. they were non-capsulated, non-mucoid, non-hyaluronic acid producing, non-iridescent, acid-agglutinable, of moderate mouse pathogenicity and usually fermented maltose and trehalose but not xylose, sorbitol or mannitol. Likewise the three strains from cat bites or scratches resembled healthy cat strains in being non-capsulated, non-iridescent and acid-agglutinable yet highly mouse pathogenic, and fermenting xylose, sorbitol and mannitol.

The strains from internal disease and from healthy throats comprised a more interesting group. Cultures 149 and 170 had properties which suggested they might have originated from cats, while 84, 275, 709 and 710 resembled typical bovine strains (capsulated, iridescent, acid-inagglutinable, highly mouse-pathogenic and fermenting xylose, sorbitol and mannitol but not maltose or trehalose). Strain 712 (from sputum) and 276 (healthy throat)

TABLE 2

PROPERTIES OF *PAST. SEPTICA* STRAINS FROM HUMAN BEINGS

| *Property examined* | *Source of strain and strain number* | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Dog bite* | | | | | *Cat bite or scratch* | | | *Internal disease* | | | | | | *Healthy throat* | |
| | *136* | *201* | *202* | *679* | *711* | *207* | *612* | *696* | *84* | *149* | *170* | *709* | *710* | *712* | *275* | *276* |
| Capsule .. .. .. | — | — | — | — | — | — | — | — | + | — | — | + | + | + | + | + |
| Mucoid colonies.. .. | — | — | — | — | — | — | — | — | — | — | — | — | — | + | — | + |
| Hyaluronic acid .. | — | — | — | — | — | — | — | — | — | — | — | — | — | + | — | + |
| Iridescence .. .. | — | — | — | — | — | — | — | — | + | — | — | — | + | + | + | + |
| Acid agglutinability .. | + | + | + | + | + | + | + | + | — | + | + | — | — | — | — | — |
| Mouse pathogenicity .. | + | + | + | ++ | ++ | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ | ++ |
| Fermentation of: | | | | | | | | | | | | | | | | |
| Xylose .. .. | — | — | — | — | + | — | + | + | + | + | + | + | + | + | + | + |
| Sorbitol .. .. | — | — | — | — | + | + | + | + | + | + | + | + | + | + | + | + |
| Mannitol .. .. | — | — | — | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Maltose .. .. | + | — | + | + | + | + | — | — | — | — | + | — | — | — | — | — |
| Trehalose .. .. | + | + | + | — | + | — | — | — | — | — | — | — | — | — | — | — |

No gas was produced in any fermentation test. All strains fermented glucose, mannose, galactose, fructose, sucrose and glycerol; none fermented arabinose, starch, dextrin, glycogen, inulin, dulcitol, erythrytol or inositol. Strain 711 fermented rhamnose, 170 and 711 raffinose, and 679, 170 and 711 lactose.

produced mucoid colonies containing hyaluronic acid, a property regularly shown by pig strains in the previous study.

## DISCUSSION

Disease in human beings associated with *Past. septica* infection is probably not rare. Schipper (1947) reviewed the world literature and found reports of 39 cases. Two categories may be recognised. The first consists of local infections following bites inflicted by cats, less frequently dogs and, on rare occasions, other animals; recovery may be slow and the condition is sometimes complicated by necrosis of underlying bone. The origin of the infection is no problem since the organism occurs widely in the mouth and throat of healthy dogs and cats and, as shown above, cultures obtained from bites have certain properties which also characterise strains from healthy dogs and cats. The comparative rarity of dog bite infections may be explained by the rough nature of the strains commonly present in the canine upper respiratory tract (Smith, 1958).

The second group consists of internal infections, the respiratory tract being most often involved. Many isolations have been made from patients with bronchiectasis (Needham, 1948; Bezjak and Mimica, 1952; Olsen and Needham, 1952; Cawson and Talbot, 1955) and the facial sinuses may also be the seat of infection (Bartley and Hunter, 1947). Other cases have involved the meninges, the pleura, the alimentary tract and the joints (von Boèr, 1917; Debré, 1919; Lévy-Bruhl, 1934; Hadorn, 1938; Ludlam, 1944; Pizey, 1953). Often there is no history of contact with animals, and this has led to suggestions that the organism may at times inhabit the human respiratory tract without causing symptoms (Regamey, 1938; Allott, Cruickshank, Cyrlas-Williams, Glass, Meyer, Straker and Tee, 1944; Olsen and Needham, 1952). There is one recorded instance of this: Straker, Hill and Lovell (1939) recovered the organism at intervals over a period of one year from the nose of a healthy laboratory worker. This appears to be the case mentioned without details by Wilson and Miles (1946), and much quoted by later writers. The present results show that *Past. septica* may occasionally colonize the throat of persons exposed to infection from animal sources and may reside there for varying periods without causing symptoms. Two such carriers were found in a group of 71 veterinary students; one carried what appeared to be a bovine, the other a porcine strain. Since the students were at the time in contact with both large and small animal material, this may indicate that farm animal strains of *Past. septica* are more likely to infect the human subject than strains from dogs and cats.

Four of the six cultures from internal infections also appeared to be farm animal strains; the other two may have been derived from cats. It is of interest that in one case there was an unequivocal history of animal contact, for strain 712 was isolated from the

sputum of a meat porter (Beck, 1956). A small but significant proportion of the reported cases of human pasteurellosis have been in farmers and other persons with ample animal contact, and there seems little doubt that infection in such cases of internal disease is usually acquired from farm animals, from meat and offal, and occasionally perhaps from household cats. Cases may also occur when *Past. septica* residing in the nose or throat of hitherto healthy carriers invades a temporarily diseased organ, such as an inflamed frontal sinus, and by multiplying there, aggravates the condition.

## CONCLUSIONS

*Past. septica* was isolated from the throat of two out of 71 veterinary students. Certain properties of the strains obtained suggested that they may have come from cattle or pigs.

*Past. septica* cultures from dog bites (7) in humans showed properties characteristic of strains from healthy dogs, and those from cat bites (3) resembled strains from healthy cats.

The characteristics of six strains from internal disease in humans suggested that the infection may have been acquired from cattle, pigs or, in two cases, cats, but not from dogs.

## ACKNOWLEDGMENTS

Special thanks are due to those members of the medical profession who sent cultures and to Mr. F. Haines for technical assistance.

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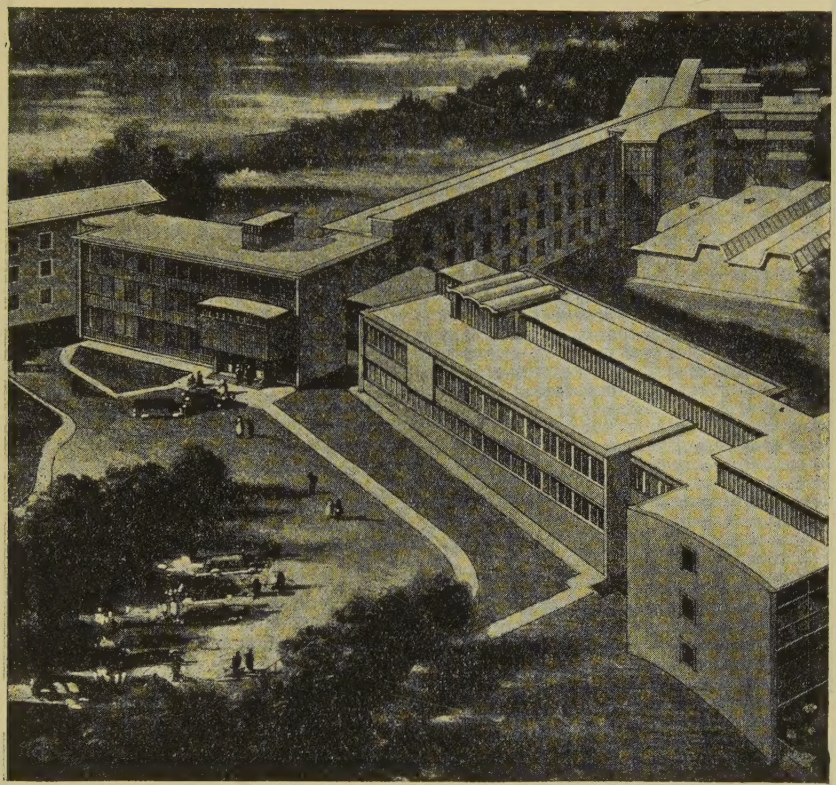
[*Received for publication, September 22nd,* 1958]



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